Université de Montpellier II

Habilitation à Diriger des Recherches

Eric GIRAUD

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PLAN

CURRICULUM VITAE

NOM	**GIRAUD Eric**
DATE DE NAISSANCE	8 Octobre 1964
NATIONALITE	Française
SITUATION DE FAMILLE	Marié, 2 Enfants
ADRESSE	Laboratoire des Symbioses Tropicales et Méditerranéennes (LSTM). Campus de Baillarguet TA10/J, 34398 Montpellier Cedex Tel. 04-67-59-37-83, Fax: 04-67-59-38-02
STATUT PROFESSIONNEL	Ingénieur de Recherche 2ème Classe

TITRES ET DIPLOMES

Thèse de Doctorat de 3ème cycle en Microbiologie et Biologie Cellulaire. Sujet : Contribution à l'étude de *Lactobacillus plantarum* amylolytique isolée du manioc fermenté. Thèse de Doctorat, Université de Provence, Aix-Marseille I. Mention: Très Honorable (1993)

Diplôme d'Etudes Approfondies en Microbiologie et Biologie Cellulaire. Réalisé durant mon Service militaire en tant que Scientifique du Contingent. Sujet : Etude de deux glycoprotéines de surface de *Plasmodium falciparum*. Université de Provence, Aix-Marseille III, Mention Assez Bien (1988)

Ingéniorat en Microbiologie Industrielle et Appliquée. Université de Provence, Aix-Marseille I - Mention Bien. (1987)

Maîtrise de Sciences et Techniques de Microbiologie Industrielle et Appliquée. Université de Provence, Aix-Marseille I- Mention Bien. (1986)

D.E.U.G. B option biochimie. Université Aix Marseille I- Mention Bien. (1984)

EXPERIENCES PROFESSIONNELLES

1988-1989 : **Ingénieur de la société CALLIOPE** (Béziers), Travaux effectués au Laboratoire de Biotechnologie du Centre O.R.S.T.O.M. de Montpellier. Sujet : Production de spores de champignons filamenteux en vue d'une application phytosanitaire.

1989 -1996: **Ingénieur d'Etude** à l'IRD - Laboratoire de Biotechnologie du Centre de Montpellier. -Etude physiologique, enzymologique et génétique des bactéries lactiques isolées des fermentations traditionnelles végétales.

Depuis le 1er janvier 1997: affectation au Laboratoire des Symbioses Tropicales et Méditerranéennes de Montpellier (LSTM). Caractérisation des mécanismes symbiotiques Légumineuses/Rhizobium

1999 : Accession au grade d'**Ingénieur de Recherche**

PARCOURS DE RECHERCHE

Issu de la filière scientifique, j'ai suivi une formation spécialisée en Microbiologie Industrielle et Appliquée, me destinant initialement à un métier dans le domaine des Biotechnologies.

Fraîchement diplômé, je suis recruté par la société CALLIOPE qui m'affecte à l'IRD (ex-ORSTOM) en 1987, dans le cadre d'une convention ORSTOM/CALLIOPE afin d'étudier la faisabilité de l'industrialisation d'un procédé de production de spores breveté par l'ORSTOM. En 1988, Maurice Raimbault, responsable du Laboratoire de Biotechnologie du centre IRD de Montpellier, me propose de rejoindre son équipe en m'offrant un contrat à durée déterminée. Mon travail de recherche s'insère alors dans le cadre d'un projet CEE STD2 intitulé : « Amélioration de la qualité des aliments fermentés à base de manioc ». Il s'agissait plus particulièrement d'étudier et de caractériser les bactéries lactiques associées aux fermentations traditionnelles afin de maîtriser cette étape primordiale dont découle directement la qualité des aliments.

Mes travaux de recherche se sont focalisés principalement sur l'étude physiologique et enzymologique d'une souche de *Lactobacillus plantarum* présentant la capacité unique d'être amylolytique. En parallèle, j'étudie la résistance des bactéries lactiques aux cyanures et nous montrons pour la première fois que les bactéries lactiques interviennent dans la détoxication du manioc, grâce à leur capacité à dégrader la linamarine (principal composé toxique du manioc). L'ensemble de ces résultats me permet de soutenir en 1993, une thèse de Doctorat de Troisième cycle. Il est à noter que durant cette période, j'accède en 1992 à un poste d'Ingénieur d'Etude de l'IRD.

De 1994 à 1996, mes recherches se portent sur la caractérisation du gène d'α-amylase de *L. plantarum* A6, afin de mieux comprendre l'origine de cette propriété retrouvée chez ce microorganisme. La caractérisation de ce gène révèle une structure particulièrement dont on a montré par la suite qu'elle intervenait dans l'attachement de l'enzyme au grain d'amidon.

C'est durant ces 2 années que je me forme aux techniques de biologie moléculaire et je découvre et m'investis avec enthousiasme dans un domaine de recherche, plus fondamental, axé sur la compréhension de certains mécanismes moléculaires.

En 1997, je réoriente ainsi mes activités de recherches sur le programme « Fixation symbiotique de l'azote », dans le cadre de la création du Laboratoire de Symbioses Tropicales et Méditerranéennes (LSTM) à Montpellier. Je participe alors à l'installation de cette nouvelle UMR, dirigé par Bernard Dreyfus, et prend en charge le développement des activités de biologie

moléculaire qui y sont développées. Je participe aux développements de plusieurs axes de recherches de ce laboratoire portant sur l'étude des mécanismes symbiotiques Rhizobium/légumineuse. En 1999, j'accède au grade d'Ingénieur de Recherche et prend la responsabilité d'une équipe dont le sujet d'étude porte sur les mécanismes moléculaires de l'adaptation des *Bradyrhizobium* photosynthétiques à leur(s) environnement(s).

Le mémoire présenté ici résume différents aspects de mes activités menées depuis 1988 ainsi que le projet de recherche que je me propose de développer au cours des 5 prochaines années.

1. DIFFUSION DE L'INFORMATION SCIENTIFIQUE

1.1 Publications avec Comité de lecture

Revue	Nombre	Impact factor
Nature	1	29,491
PNAS	1	10,26
J. Bacteriol.	2	3,712
Appl. Environ. Microbiol	4	3,541
Microbiology	1	2,7
Gene	1	2,258
FEMS Microbiol. Lett.	1	1,673
Appl. Microbiol.Biotechnol.	1	1,641
J. Appl. Bacteriol	1	1,599
J. Appl. Microbiol.	1	1,521
J. Sci. Food Agr.	1	1,097
Can. J. Microbiol.	1	1,072
Biotechnol. Lett.	1	0,916
Biotechnol. Tech.	1	0,613

Soit 18 publications de rang A dont 10 en premier auteur, 2 en dernier auteur et 3 en second auteur.

1- Giraud E, Lelong B et Raimbault M. (1991) Influence of pH and initial lactate concentration on the growth of *Lactobacillus plantarum.* Applied and Microbiology Biotechnology. **36** : 96-99.

2- Brugidou C, Rocher A, **Giraud E**, Lelong B, Marin B et Raimbault M. (1991) A new high performance liquid chromatographic technique for separation and determination of adenilyc and nicotinamide nucleotides in *Lactobacillus plantarum.* Biotechnology Techniques. **5** : 475-478.

3*- Giraud E, Brauman A, Keleke S, Lelong B et Raimbault M. (1991) Isolation and physiological study of an amylolytic strain of *Lactobacillus plantarum.* Applied and Microbiology Biotechnology. **36** : 379-383.

4- Giraud E, Gosselin L et Raimbault M. (1992) Degradation of the cassava linamarin by lactic acid bacteria. Biotechnology Letters. **14** : 593-598.

5*- Giraud E, Gosselin L et Raimbault M. (1993) Production of a *Lactobacillus plantarum* starter with linamarase and amylase activities for cassava fermentation. Journal of the Science of food and Agriculture. **62** : 77-82.

6*- Giraud E, Gosselin L, Marin B, Parada J. L, et Raimbault M. (1993) Purification and characterization of an extracellular amylase activity from *Lactobacillus plantarum* strain A6. Journal of Applied Bacteriology. **75** : 276-282.

7*- Giraud E, Champailler A et Raimbault M. (1994) Degradation of raw starch by a wild amylolytic strain of *Lactobacillus plantarum*. Applied and Environmental Microbiology. **60** : 4319-4323.

8*- Giraud E et Cuny G. (1997) Molecular characterization of the α-amylase genes of *Lactobacillus plantarum* A6 and *Lactobacillus amylovorus* reveals an unusual 3' end structure with direct tandem repeats and suggests a common evolutionary origin. Gene. **198** : 149-157.

9*- Giraud E, Champailler A, Moulard S et Raimbault M. (1998) Development of a miniaturised selective strategy of lactic acid bacteria for evaluation of mixed starter in a model cassava fermentation. Journal of Applied Microbiology. **84** : 444-450.

10*- Moulouba F, Lorquin J, Willems A, Hoste B, **Giraud E**, Dreyfus B, Gillis M, de Lajudie P et Boivin C. (1999) Photosynthetic Bradyrhizobia from *Aeschynomene* are specific to stem nodulated species and form a separate 16S rDNA group. Applied and Environmental Microbiology. **65** : 3084-3094.

11- Babic I, Fischer-Le Saux M, **Giraud E** et Boemare N. (2000) Occurrence of natural dixenic associations between the symbiont *Photorhabdus luminescens* and bacteria related to *Ochrobactrum* spp. in tropical entomopathogenic Heterorhabditis spp. (Nematoda Rhabditida). Microbiology UK. 146: 709-718.

12*- Hannibal L, Lorquin J, Angles d'Ortoli N, Garcia N, Chaintreuil C, Masson-Boivin C, Dreyfus B. et **Giraud E**. (2000) Isolation and characterization of canthaxanthin biosynthesis genes from the photosynthetic *Bradyrhizobium* sp. strain ORS278. Journal of Bacteriology, **182**:3850-3853.

13*- Chaintreuil C, **Giraud E**, Prin Y, Lorquin J, Gillis M, De Lajudie P, Dreyfus B. (2000) Photosynthetic bradyrhizobia are natural endophytes of the african wild rice *Oryza breviligulata*. Applied and Environmental Microbiology. **66**: 5437-5447.

14*-Giraud E, Hannibal L, Fardoux J, Verméglio A, Dreyfus B. (2000) Effect of *Bradyrhizobium* photosynthesis on stem nodulation of *Aeschynomene sensitiva*. The Proceeedings of National Academy of Sciences USA (PNAS) **26**: 14795-14800.

15*- Chaintreuil C, Boivin C, Dreyfus B, **Giraud E**. (2001) Characterization of the common nodulation genes of the photosynthetic *Bradyrhizobium* sp. ORS285 reveals the presence of a new Insertion Sequence upstream of nodA FEMS Microbiology Letters. **194** :83-86.

16*- Sy A, **Giraud E**, Jourand P, Willem A, de Lajudie P, Samba R, Prin Y, Neyra M, Gillis M, Boivin C, Dreyfus B. (2000). Methylotrophic *Methylobacterium* nodulate and fix nitrogen in symbiosis with legumes. Journal of Bacteriology. *183*: 214-220.

17- Sy A, **Giraud E**, Samba R, De Lajudie P, Gillis M, Dreyfus B. (2001) Certain legumes of the genus Crotalaria are specifically nodulated by a new species of *Methylobacterium*. Can. J. Microbiol. **47**: 503-508.

18*- Giraud E, Fardoux J, Fourrier N, Hannibal L, Genty B, Bouyer P, Dreyfus B, Verméglio A. (2002) Phytochrome controls the photosystem synthesis in anoxygenic bacteria. Nature. **417** :202-205.

*** : Article mis en annexe**

1.2 Brevets

19- Dreyfus B, **Giraud E**, Boivin C. 1999. Nouvelles bactéries symbiotiques et leurs applications. Brevet N° 9914179. Déposé en France le 10 Novembre 1999. :

20- Hannibal L, **Giraud E**, (2001) Isolated carotenoid biosynthesis gene cluster involved in canthaxanthin production and applications thereof. Brevet déposé aux USA N° 60/297,247 le 12 juin 2001, et déposé au canada N° 2 349 040 le 13 juin 2001.

21- Giraud E, Vermléglio A (2002) Construction nucléotidiques pour la régulation de gène(s) par la lumière et leurs applications. Brevet N° 02 01 307. Déposé en France le 4 février 2002.

1.3 Chapitres d'Ouvrages

22- Giraud E. (1993) Contribution à l'étude physiologique et enzymologique d'une nouvelle souche de *Lactobacillus plantarum* amylolytique isolée du manioc fermenté. Thèse de Doctorat, Université de Provence, Aix-Marseille I, 139 p.

23- Giraud E et Raimbault M. (1995) Les bactéries lactiques et la détoxication: la dégradation de la linamarine du manioc. In: Les bactéries lactiques. Actes du colloque LACTIC 94. Caen 79 septembre 1994. Presses universitaires de Caen.

24- Giraud E, Brauman A, Keleke S, Gosselin L et Raimbault M. (1995) Contrôle de la fermentation du manioc pour un meilleur gari: utilisation d'un starter de *Lactobacillus plantarum* à activité linamarase et amylase. In: Transformation alimentaire du manioc. Eds : Tom Agbor Egde, Brauman A, Griffon D et Trêche S. ORSTOM éditions.

25- Boivin C. et **Giraud E**. 1999. Molecular symbiotic characterization of rhizobia: towards a polyphasic approach using Nod factors and *nod* genes. p 295-299. In: Highlights of Nitrogen Fixation Research. Martinez E. and Hernandez G., eds. Plenum Publishing Corporation.

26- Sy A, Jourand P, **Giraud E**, Ndoye I, De Lajudie P, Boivin-Masson C, Samba R, Neyra M, Willems A, Gillis M, Drefyus B, (2000) Multipurpose legumes of the tropical genus *Crotalaria* are associated with unusual rhizobia. In Nitrogen Fixation: From molecules to crop productivity, p. 537. Edited by F O Pedrosa, M Hungria, M G Yates, W E Newton. Kluwer Academic Publishers, Dordrecht (The Netherlands).

27- Moulin L, Debellé F, **Giraud E**, Mangin B, Dénarié J, Boivin-Masson C, (2000) The *nodA* sequence of rhizobia give clues on structural features of Nod factors. In Nitrogen fixation: from molecules to crop Productivity, p204. Edited by F.O. Pedrosa, M. Hungria, M.G. Yates & W.E. Newton. Dordrecht:

28- Giraud E, Hannibal L, Chaintreuil C, Lorquin J, Molouba F, Mylovsky S, Hurard C, Boivin C, Dreyfus B, (2000) Photosynthesis in *Aeschynomene Bradyrhizobium* sp. ORS278: Genetic analysis and role in symbiosis. In Nitrogen Fixation: From molecules to crop productivity, p. 145. Edited by F O Pedrosa, M Hungria, M G Yates, W E Newton. Kluwer Academic Publishers, Dordrecht (The Netherlands).

1.4 Conférences Orales

29- Giraud E et Raimbault M. 1991. Utilización de la cromatografia liquida de alta resolución para la caracterisación bioquimica de la fermentación del almidón de yuca. Congrès : Avances sobre almidón de yuca, 17-20 juin, CIAT, Cali, Colombie.

30- Giraud E et Raimbault M. 1991. Efecto del cianuro y degradación de la linamarina par las bacterias lacticas. Congrès: Avances sobre almidón de yuca, 17-20 juin, CIAT, Cali, Colombie.

31- Raimbault M, **Giraud E**, Saucedo G et Soccol C. 1991. Valorización de la yuca a través de la fermentación (Bacterias lacticas, levaduras y hongos filamentosos). Congrès : Avances sobre almidón de yuca, 17-20 juin, CIAT, Cali Colombie.

32- Giraud E. 1993. Traditional cassava fermentation. Symposium sur les technologies de fermentation alimentaire, Présidence de session et animation de l'atelier "Technologie de la fermentation du manioc". Dakar, 13-16 Décembre.

33- Giraud E, Brauman A, Keleke S, Gosselin L et Raimbault M. 1994. Search for lactic acid bacteria with desired physiological properties for cassava fermentation control. International Meeting on cassava Flour and Starch. 11-15 janvier, CIAT, Cali, Colombia.

34- Giraud E et Raimbault M. 1994. Les bactéries lactiques et détoxication. Un exemple: la dégradation de la linamarine du manioc. Lactic 94, 7-9 Septembre, Caen, France.

35- Raimbault M, Ramirez Toro C, **Giraud E,** Soccol C et Saucedo G**.** 1994. Fermentation process in cassava bioconversion. International Meeting on cassava Flour and Starch. 11-15 janvier, CIAT, Cali, Colombia.

36- Giraud E et Cuny G. 1997. Les gènes d'α-amylase de *Lactobacillus plantarum* A6 et *L. amylovorus* : organisation atypique avec la présence de séquences répétées en tandem. 8ème Colloque du Club des Bactéries Lactiques. Dijon, 28-30 Mai.

37- Giraud E, Hannibal L, Chaintreuil C, Lorquin J, Molouba F, Mylovsky S, Hurard C, Boivin C et B. Dreyfus. 1999. Photosynthesis in *Aeschynomene Bradyrhizobium* sp. ORS278: genetic analysis and role in symbiosis. 12th International Congress on Nitrogen Fixation. Foz do Iguaçu. Brésil, 12-17 Septembre.

38- Sy A, Jourand P, **Giraud E**, Ndoye I, de Lajudie P, Boivin-Masson C, Samba R, Neyra M, Willems A, Gillis M et Dreyfus B. 1999. Multipurpose legume of the tropical genus *Crotalaria* are associated with unusual rhizobia. 12th International Congress on Nitrogen Fixation. Foz do Iguaçu. Brésil, 12-17 Septembre.

39- Sy A, **Giraud E,** Jourand P, Garcia N, Willems A, De Lajudie P, Prin Y, Neyra M, Gillis M, Masson-Boivin C, Dreyfus B, (2000) A group of *Methylobacterium* nodulates and fixes nitrogen in symbiosis with leguminous plants. Mediterranean Conference of Rhizobiology. Montpellier, France, 9-13 Juillet 2000

40- Giraud E, (2001) La photosynthèse chez les *Bradyrhizobium* associés aux légumineuses du genre *Aeschynomene* : Analyse génétique et rôle durant la symbiose. Conférence donnée au Collège de France à Paris le 12 Juin 2001.

41- Giraud E, Fardoux J, Fourrier N, Hannibal L, Genty G, Bouyer P, Dreyfus B, Verméglio A. (2002) Lorsque la lumière aide les bactéries à s'associer avec des plantes exemple de symbiose originale : *Bradyrhizobium / Aeschynomene*. 5èmes rencontres plantes bactéries. Aussois, France, 14-17janvier 2002.

2. ACTIVITES DE FORMATION

Formation des étudiants à la recherche

THESE

Chaintreuil C. (Université de Montpellier II, Directeur de thèse : Bernard Dreyfus, Co-direction : Eric Giraud- Taux d'encadrement 70%, Thèse soutenue en Décembre 2000. Mention Très honorable). Caractérisation des rhizobia tropicaux associés aux riz sauvages. (Co-auteur des publications 12,13, 15, 28, 37). Thèse suivie d'un stage Post- Doctorale de 6 mois réalisé au LSTM sur la Production de canthaxanthine par les *Bradyrhizobium* photosynthétiques- financement Aventis. Actuellement à la recherche d'un emploi.

Sy A. (Université de Lyon Claude Bernard, Directeur de thèse : Bernard Dreyfus, Co-direction : Eric Giraud- Taux d'encadrement 70%, Thèse soutenue en Mars 2001. Mention Très honorable). Etude des rhizobiums des légumineuses des jachères. (Co-auteur de la publication 16, 17, 26, 38, 39). Thèse suivie d'un stage Post Doctorale d'un an à L'ENITIAA de Nantes sur l'etude de la microflore du saumoin fumé par TGGE. Actuellement en stage Post-Doc à l'INRA de Toulouse- Etude des bactéries methylotrophes de la phyllosphère.

DEA

Champailler A. 1994. Cultures mixtes de bactéries lactiques sur manioc : développement d'une méthode pour le suivi spécifique des différentes populations. DEA, Université de Montpellier II, 8 mois. (Co-auteur des publications 7, 9).

Ndeye F. 1998. Etude de la coévolution des légumineuses (*Crotalaria*) et des bactéries symbiotiques fixatrices d'azote associées (*Rhizobium*). DEA, Université de Montpellier II, 6 mois.

Villegas C. 1999. Recherche de gènes bactériens responsables de la spécificité Rhizobium-légumineuse. DEA Université de Lyon Claude Bernard , 6 mois.

Fourrier N. 2001. Régulation de la biosynthèse de l'appareil photosynthétique par la lumière *via* un bactériophytochrome chez *Bradyrhizobium* ORS278. DEA, Université de Montpellier II, 8 mois. (Co-auteur de la publication 18).

Jaubert M. 2002-2003. Contrôle de la photosynthèse par un phytochrome chez une bactérie symbiotique : caractérisation de la chaîne de transduction du signal lumineux. DEA, Université de Montpellier II en cours

Ingénieur

Montalti P. 1990. Faisabilité de l'industrialisation d'un procédé de production de spores de *Beauveria bassiana.* Rapport de fin d'études à l'Ecole Nationale Supérieure des Techniques Industrielles et des Mines d'Alès, 6 mois.

Nicaud I. 1990. Etude de la sporulation de *Beauveria bassiana.* Rapport de fin d'études à l'Ecole National Supérieure des Techniques Industrielles et des Mines d'Alès, 6 mois.

Gosselin L. 1992. Effet d'un starter de *Lactobacillus plantarum* à activités linamarase et amylase sur la fermentation du manioc pour la production de gari. Stage ingénieur de fin d'études. Université de Technologie de Compiègne, 6 mois. (Co-auteur des publications 4, 5, 6, 24, 33, 48).

Moulard S. 1994. Co-cultures de bactéries lactiques sur manioc : mise au point d'une technique miniaturisée de dénombrement permettant le suivi spécifique des différentes populations. Stage Ingénieur de fin d'études, INSA-Lyon, 6 mois. (Co-auteur de la publication 9).

Hurard C. 1998. Caractérisation des gènes de photosynthèse (opéron *puf*) chez un *Bradyrhizobium* photosynthètique. Ingénieur en Congés Individuel de Formation, 9 mois. (Co-auteur de la publication 37).

Anglès d'Ortoli N. 1998. Caractérisation des gènes de biosynthèse de la canthaxanthine chez *Bradyrhizobium* ORS278. Stage d'Ingénieur de fin d'Etude. Ecole ESIL-Département GBMA (Marseille), 6 mois . (Co-auteur de la publication 12).

Licence-Maîtrise:

Rocher A. 1990. Etude du métabolisme énergétique des bactéries lactiques. Maîtrise de Sciences et Techniques, Université Aix-Marseille I, 2 mois. (Co-auteur de la publication 2).

Garcia N. 1999. Caractérisation des gènes de biosynthèse de la spirilloxanthine de *Bradyrhizobium* ORS278. Stage de Maîtrise Université de Montpellier II, 8 mois. (Co-auteur de la publication 12).

Jacolot MF. 1999. Etude du caractère fixateur d'azote libre de la souche *Bradyrhizobium* ORS278. Caractérisation des gènes *nif*, obtention de mutant. Stage de Maîtrise Université de Bretagne Occidentale. 6 mois.

Jaubert M. 2002. Surproduction et purification d'un bactériophytochrome recombinant chez *E. coli.* Stage de Maîtrise Université de Montpellier II, 3 mois.

3. PARTICIPATION A DES PROJETS ET MISSIONS

Projets

Projet ANVAR : Production de spores de *Beauveria bassiana* (1990)

Projet CEE STD2 : Amélioration de la qualité des aliments fermentés à base de manioc. Contrat TS2A-0226.(1989-1992)

Projet CEE STD3: Valorisation des produits, sous produits et déchets de la petite et moyenne industrie du manioc en Amérique Latine. Contrat TS3-CT92-010. (1993-1996)

Projet ALLIANCE: Développement de méthodes de caractérisation symbiotique des rhizobia basées sur l'analyse des facteurs Nod et des gènes de nodulation ». Financement pour un projet de coopération bilatérale. Programme Alliance (France-Angleterre). Ministère des Affaires étrangères. 1998.

AIP Microbiologie INRA: Bases moléculaires de la diversité des propriétés symbiotiques des rhizobia: application à la classification et à l'étude de l'évolution et de l'adaptation à la plante-hôte». AIP Microbiologie INRA. 1998-1999. INRA-Dijon, LSTM-Montpellier, ORSTOM-Dakar.

Programme National : Dynamique de la Biodiversité et environnement dans le cadre du programme CNRS : (Biodiversité et écologie des interaction durable) **:** «Evolution des *rhizobia* en réponse à un environnement particulier: le modèle des *Bradyrhizobium* photosynthétiques d'*Aeschynomene* ».

Missions

1991 Mission d'un mois au CIAT (Centre International d'Agronomie Tropicale) de Colombie. Présentation des résultats obtenus durant le contrat CEE STD2. Mises au point des protocoles d'isolement et de caractérisation de la microflore lactique amylolytique du manioc aigre.

1992. Mission d'un mois au Laboratoire de Microbiologie ORSTOM de Brazzaville. Essais réels d'inoculation du manioc roui avec *L. plantarum* A6. Formation du personnel local aux techniques de fermentations liquides et dosage des cyanures.

1993. Mission d'un mois au Laboratoire de Bioconversion. Universidad del Valle-Cali-Colombie. Présentation des résultats au congrès organisé par le CIAT. Formation du personnel local aux techniques de numération des bactéries en culture mixte.

1996. Mission d'un mois au Laboratoire de Microbiologie de Dakar. Elaboration d'un nouveau programme de Recherche sur l'étude des mécanismes symbiotiques légumineuses/rhizobium.

4 ACTIVITE DE RECHERCHE (1988-2002)

4.1 ETUDE DES BACTERIES LACTIQUES ASSOCIEES AUX FERMENTATIONS TRADITIONNELLES DU MANIOC (1988-1996)

Le manioc (*Manihot esculenta* Crantz) constitue l'aliment de base de plus de 500 millions de personnes (Cock, 1982). Il est considéré comme un élément clé de la lutte contre la faim dans les pays africains et cela, malgré une certaine toxicité liée à la présence de glucosides cyanogéniques (linamarine essentiellement) et le caractère hautement périssable des racines après la récolte. Les populations autochtones ont empiriquement élaboré divers procédés permettant de stabiliser ce produit et de réduire sa toxicité. La fermentation que l'on retrouve dans la quasi-totalité de ces divers procédés est une étape importante tant pour l'obtention des qualités organoleptiques requises que pour la détoxication du manioc.

Le problème majeur de l'ensemble de ces procédés artisanaux réside dans la qualité très fluctuante des différents aliments ainsi obtenus. En effet, le processus fermentaire qui s'effectue spontanément grâce au développement de la microflore épiphyte peut conduire à des produits d'une qualité organoleptique, microbiologique ou toxicologique indésirable. Les mesures permettant de réduire cette variabilité impliquent avant tout de comprendre les phénomènes biochimiques, enzymologiques et microbiologiques qui interviennent. Ceci devrait permettre dans un deuxième temps de maîtriser l'étape de fermentation et de conduire à une standardisation de la qualité des produits obtenus.

C'est dans ce cadre qu'un projet CEE intitulé « Amélioration de la qualité des aliments fermentés à base de manioc » a été mis en place. Ce programme avait pour finalité d'apporter des solutions technologiques permettant d'assurer la fabrication d'aliments de bonne qualité.

4.1.1 Etude physiologique de *Lactobacillus plantarum*.

Les bactéries lactiques jouent un rôle prépondérant durant le processus de fermentation naturelle du manioc. La baisse rapide de pH et la production d'acide lactique provoquées par leur croissance vont contribuer grandement à la conservation et à l'obtention des caractéristiques organoleptiques des différents aliments obtenus : l'acide lactique, outre son effet inhibiteur sur la croissance de nombreux microorganismes pathogènes, est en effet reconnu comme le principal agent responsable de la flaveur de nombreux produits.

Sur la base de ces considérations, nous avons étudié les caractéristiques métaboliques d'un ensemble de souches de collection appartenant aux quatre principaux genres de bactéries lactiques. *Lactobacillus plantarum* est apparu rapidement comme l'espèce la plus intéressante.

En effet, elle est homolactique, elle a une croissance rapide, elle est acido-tolérante et permet de diminuer rapidement le pH en dessous de 4. D'autre part, elle excrète de fortes quantités d'acide lactique et donne, dans les conditions étudiées, les meilleurs rendements en biomasse. Nous avons observé que les rendements de fermentation obtenus avec cette souche étaient très élevés, mais que la concentration en biomasse restait limitée car au-delà d'une concentration en substrat de 50 g/l, les bactéries sont l'objet d'un découplage énergétique et transforment quantitativement le glucose en acide lactique.

Par ailleurs, nous avons étudié l'effet du pH et du lactate (2 des principaux paramètres des fermentations naturelles) sur les rendements de croissance et de production de lactate par *L. plantarum*. Une publication de rang A a été rédigée sur la base de ces résultats (1). Cette étude a été complétée par la mise au point d'une nouvelle technique HPLC permettant l'analyse du pool nucléotidiques en fonction des différents états physiologiques de la souche. La description de cette technique a donné lieu à une autre publication de rang A (2).

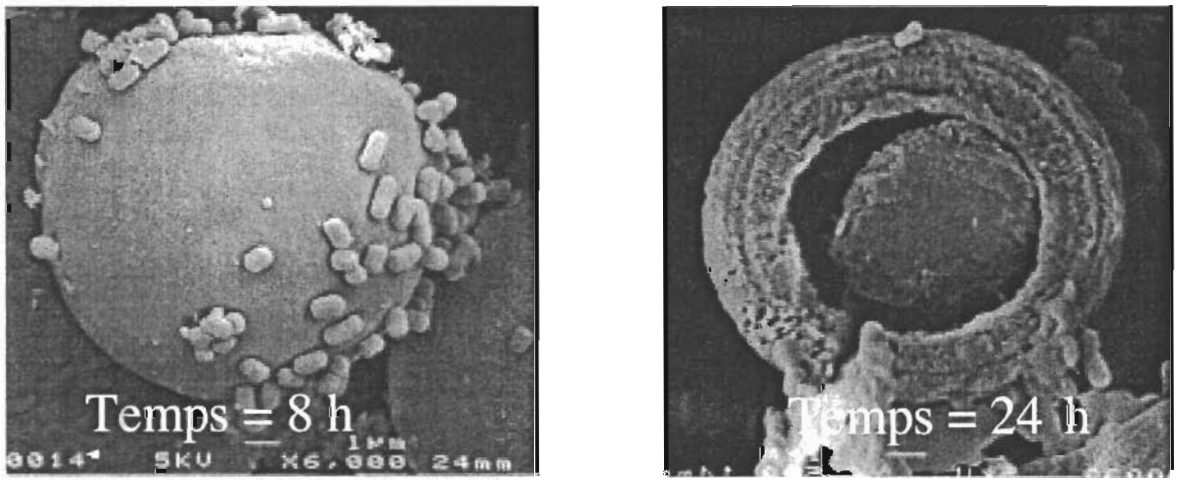
Durant cette étude, j'ai encadré le travail de stage d'une étudiante en Maîtrise (Anne Rocher). J'avais également la responsabilité d'un technicien, Bertrand Lelong.

4.1.2 Isolement et caractérisation d'une souche de *Lactobacillus plantarum* (A6) amylolytique.

Les racines de manioc étant constituées essentiellement d'amidon (plus de 86% de la matière sèche), la sélection d'une bactérie lactique capable de métaboliser ce composé semblait particulièrement intéressante. En effet, l'inoculation du manioc avec une telle souche permettrait d'augmenter les teneurs finales d'acide lactique dans les aliments à base de manioc, mais surtout lui permettrait de se développer et de s'imposer par rapport à des souches non amylolytiques. Cependant, la capacité à hydrolyser l'amidon est une caractéristique peu fréquente chez les bactéries lactiques. Les principales souches identifiées comme telles, sont : *Streptococcus bovis*, *S. equinus*, *Lactobacillus amylophilus, L. amylovorus*, *L. acidophilus, L. cellobiosus* (Cotta, 1988).

Nous avons, en collaboration avec Alain Brauman, alors Chargé de Recherche au laboratoire de Microbiologie du Centre ORSTOM de Brazzaville, et le Professeur Jose luis Parada de l'Université d'Argentine, mis au point des milieux sélectifs permettant l'isolement de bactéries lactiques amylolytiques. Très rapidement une des souches testées a particulièrement retenu notre attention en raison de son fort pouvoir amylolytique. Elle a été identifiée sur la base de critères phénotypiques (métabolismes des sucres, température de croissance, inhibiteur de croissance, profils SDS PAGE des protéines...) et sur la base de critères génétiques (analyse de la séquence d'une région variable du 16S RNA) comme une souche de *Lactobacillus plantarum*

(souche A6). C'était la première fois que l'on décrivait une telle propriété amylolytique chez un *Lactobacillus plantarum*. Une publication de rang A en collaboration Alain Brauman a été réalisée décrivant l'isolement et la description de cette souche (3). Cette souche s'avéra être particulièrement intéressante. En effet, outre son caractère amylolytique tout à fait unique, les quantités d'amylase excrétées sont telles que la souche conserve des taux de croissance identiques sur glucose et amidon. Nous avons de plus démontré par la suite que ce microorganisme était capable de dégrader entièrement les grains d'amidon brut. Lorsque les conditions de culture sont contrôlées (régulation du pH), la bactérie peut ainsi transformer complètement en acide lactique, les 100g/litre de farine de manioc initialement présents. Cette capacité de *L. plantarum* A6 à dégrader l'amidon, sans aucun prétraitement physico-chimique, pouvait être particulièrement intéressante à considérer pour une production industrielle d'acide lactique à partir d'amidon cru. Un article a été publié dans Applied and Environmental Microbiology sur la base de ces résultats (7).



Dégradation des grains d'amidon par la souche L. plantarum A6 à différents temps de fermentation

Etant donnée la propriété de la souche *L. plantarum* A6 à dégrader l'amidon, et le peu d'études effectuées sur les activités amylolytiques des bactéries lactiques, il nous est apparu nécessaire également de caractériser cette enzyme.

L'α-amylase excrétée en grande quantité par la souche a été ainsi purifiée et ses propriétés physico-chimiques ont été déterminées. Les résultats de cette étude, conduite avec un stagiaire de fin d'Etudes de l'UTC (Laurent Gosselin), ont été publiés dans une revue de rang A (6).

Par rapport aux caractéristiques des autres amylases bactériennes décrites dans la littérature, les propriétés de l'enzyme synthétisée par *L. plantarum* A6 apparaissaient être très proches de celles de *Bacillus subtilis*: enzyme extracellulaire, pH optimum identique (pH 5.5),

température optimale identique (T° 55°C), intervention de résidus de tryptophane et de tyrosine lors de la catalyse et présence de formes multiples (agrégats).

La capacité de la souche *L. plantarum* A6 à dégrader l'amidon, nous a conduit à nous interroger sur l'acquisition de cette propriété. Pourrait-elle résulter d'un transfert de matériel génétique entre *B. subtilis* et *L. plantarum,* deux microorganismes de la microflore naturelle du manioc fermenté dont nous avons observé que les activités amylolitiques présentaient de fortes similitudes. Des études plus approfondies ont été conduites par la suite sur la caractérisation du gène d'α-amylase de *L. plantarum* A6 pour répondre à cette question.

4.1.3. Caractérisation des gènes d'α -amylase de *L. plantarum* A6 et *L. amylovorus*

Cette étude a été réalisée en collaboration avec Gérard Cuny (Directeur de Recherche au laboratoire de génétique moléculaire des parasites et des vecteurs-ORSTOM). J'ai pu être formé, grâce à son aide, aux techniques de base utilisées en biologie moléculaire (PCR, clonage, Southern blot, séquençage...).

Le gène d'α-amylase (*amyA*) de *L. plantarum* A6 a pu être isolé du génome bactérien par PCR en utilisant des amorces dégénérées, définies à partir de la séquence en aminoacides de peptides résultant de la digestion trypsique de l'enzyme purifiée. Cette étape de séquençage de la protéine a été réalisée en collaboration avec le Dr Derancourt du CRBM (CNRS-Montpellier).

L'analyse de la séquence nucléotidique montre un cadre de lecture de 2739 pb codant pour une protéine de 913 aminoacides correspondant à un poids moléculaire de 99,5 kDa. Le gène (*amyA*) s'organise en 2 régions principales de taille similaire. L'extrémité NH2 (480 premiers aminoacides) présente les caractéristiques typiques de la famille des α-amylases. Les plus fortes similarités sont les suivantes : 96,8 % d'homologie sur 155 aa avec la séquence partielle disponible de l'α-amylase de *L. amylovorus* et 64.5 % d'identité sur 462 aa avec l'α-amylase de *B. subtilis*. L'extrémité COOH présente quant à elle, une structure atypique, jamais décrite à ce jour. Elle est constituée de 4 séquences répétées en tandem de 104 aa, présentant 100% d'homologie entre elles. Ceci suggère un évènement d'insertion dans un gène ancestral d'α-amylase d'une séquence de 312 nucléotides, suivi de duplications successives.

Etant donnée la très forte similarité que nous avons observée au niveau de l'extrémité N-terminal du gène *amyA* de *L. plantarum* A6 avec la séquence partielle disponible du gène d'α-amylase de *L. amylovorus*, nous avons été amenés à caractériser entièrement ce dernier afin de préciser le degré exact d'homologie entre ces 2 gènes.

L'analyse de la séquence nucléotidique du gène (*amyA*) de *L. amylovorus* montre un cadre de lecture de 2862 bp codant pour une protéine de 954 aa. Une parfaite homologie est observée entre les 2 gènes au niveau de l'extrémité 5' avec seulement 7 mis-appariements sur 1600 nucléotides. L'extrémité 3' montre aussi la présence de séquences répétées en tandem, mais des différences majeures sont observées : (i) l'addition d'une séquence répétée (5 au lieu de 4), (ii) une unité de répétition plus courte (91 aa au lieu de 104 aa).

Par ailleurs les très fortes homologies structurales retrouvées entre les 2 gènes suggèrent que ceux-ci dérivent d'un ancêtre commun, et pourraient avoir évolué indépendamment par des événements de duplication et de recombinaison. Puisque ces séquences répétées sont parfaitement conservées à l'intérieur d'une souche et très bien préservées entre les 2 souches, il peut être suggéré qu'un tel événement est relativement récent.

Ces résultats ont été publiés dans la revue Gene (8), par ailleurs une conférence a été donnée sur ce sujet lors du 8ème colloque du Club des Bactéries Lactiques.

Il a pu être montré par la suite, par une étudiante qui a repris le sujet après mon départ, que ces séquences répétées permettaient la reconnaissance et l'adhésion de l'α-amylase sur le grain d'amidon (Rodriguez Sanoja *et al.* 2000), ce qui pouvait expliquer la capacité de cette enzyme à dégrader l'amidon brut.

4.1.4 Les bactéries lactiques et la détoxication du manioc

Les racines de manioc contiennent en forte quantité deux glucosides cyanogéniques : la linamarine et la lotaustraline, qui leur confèrent une toxicité élevée (Wood, 1965). Bien que la majorité de ces composés toxiques soit éliminée au cours des différentes étapes de transformations, une quantité résiduelle peut être retrouvée dans certains aliments, selon le procédé de fabrication utilisé. Une consommation journalière de ces derniers aliments peut donc entraîner une toxicité chronique se manifestant par des problèmes neurologiques et métaboliques.

Durant la préparation du gari (aliment fermenté à base de manioc), deux étapes semblent essentielles pour assurer une bonne détoxication (Ikediobi & Onyike, 1982): a) l'étape de râpage qui permet par l'endommagement de la structure cellulaire végétale, la libération d'une linamarase capable d'hydrolyser la linamarine en glucose et en acétone cyanohydrine, b) l'étape de cuisson qui permet une volatilisation des cyanures libres.

Le rôle de l'étape de fermentation et en particulier des bactéries lactiques dans ce processus de détoxication n'avait jamais été jusqu'alors clairement démontré.

Dans cette étude, notre objectif a été de déterminer d'une part, si les bactéries lactiques qui constituent la flore prédominante dans le processus de fermentation avaient une activité linamarase et d'autre part, de contribuer à une meilleure compréhension des mécanismes de dégradation des glucosides cyanogéniques par ces bactéries.

Nous avons pu mettre en évidence, au cours de cette étude, une bonne résistance des bactéries lactiques aux cyanures, démontrant ainsi que ces microorganismes étaient très compétitifs et particulièrement bien adaptés aux milieux à base de manioc. Par ailleurs, nous avons mis en évidence que la plupart des bactéries lactiques pouvaient dégrader la linamarine. La souche *L. plantarum* A6 présente la plus forte activité linamarase mesurée et son niveau de production peut être fortement stimulé par le cellobiose.

L'ensemble de ces travaux a été publié dans une revue de rang A (4). Par ailleurs, 2 conférences ont été données sur ces résultats et plusieurs posters ont été présentés à des congrès nationaux et internationaux.

4.1.5 Utilisation des bactéries lactiques comme starter de la fermentation du manioc

Nous avons montré au cours des études précédentes, les potentialités que pouvaient offrir la souche *L. plantarum* A6: souche acido-tolérante, produisant de l'acide lactique en forte quantité, ayant un taux de croissance élevé avec un rendement en biomasse/substrat important, capable de dégrader l'amidon brut ainsi que la linamarine. Bien qu'elle ne produise pas majoritairement l'isomère L(+) lactate, la souche présente la plupart des critères requis pour être utilisée en tant que starter efficace de la fermentation du manioc en vue de la production de gari.

Cependant, nous avons observé une influence notable du substrat carboné sur la biosynthèse de l'amylase et de la linamarase. L'amidon apparaît comme le substrat carboné permettant une production maximale d'amylase tandis que le cellobiose permet une production maximale de linamarase. Par ailleurs, les conditions de conservation du microorganisme ont une influence sur le maintien de cette dernière activité enzymatique.

Ici, notre objectif a été d'une part, d'étudier la physiologie de *L. plantarum* A6 en vue de produire un starter dans lequel la souche serait dans un état physiologiquement actif, produisant conjointement une activité amylase et linamarase, et d'autre part, d'étudier l'effet d'un tel inoculum sur la fermentation naturelle du manioc.

Nous avons ainsi observé que la biosynthèse de ces 2 enzymes était réprimée par le glucose. Sur un milieu à base de cellobiose, la souche présente au contraire une forte activité linamarase et amylase.

L'utilisation de cette bactérie comme starter de la fermentation du manioc, dans un procédé type gari, semble améliorer le profil d'acidification et nous permet d'envisager de réduire la durée de la fermentation à 24 h. Cependant, cela ne semble pas apporter d'amélioration significative au niveau de la détoxication du manioc, et présente même un effet antagoniste.

En ce qui concerne les autres paramètres physico-chimiques et microbiologiques, il apparaît que l'inoculation du manioc avec une souche de *L. plantarum* entraîne: le passage d'un profil fermentaire hétérolactique caractéristique de la fermentation naturelle, à un profil homolactique; une baisse plus importante et rapide du pH (pH final: 3,8); une production supérieure d'acide lactique. Le caractère amylolytique de *L. plantarum* A6 permet d'augmenter la teneur finale en acide lactique jusqu'à 5 g /100 g de matière sèche.

Ces derniers points suggèrent que l'utilisation de *L. plantarum* A6 comme starter pourrait jouer un rôle significatif dans le développement des qualités organoleptiques, mais aussi dans la standardisation et la préservation du produit final obtenu, grâce aux fortes quantités d'acide lactique produites par la souche et la baisse plus rapide et importante du pH qui en résulte. Ces résultats ont été publiés dans une revue de rang A (5).

Cependant l'utilisation d'une seule souche semble trop restrictive pour l'obtention d'un aliment présentant tout un panel de caractéristiques organoleptiques. L'emploi d'un mélange de microorganismes présentant des propriétés physiologiques et métaboliques complémentaires constitue probablement la meilleure approche pour obtenir un aliment ayant des propriétés nutritionnelles et sanitaires désirées. Aussi, afin de mieux étudier l'écophysiologie des fermentations mixtes et évaluer les effets de starters composés, nous avons proposé l'utilisation de conditions modèles de fermentation du manioc associée à une nouvelle stratégie de dénombrement des différentes populations. Cette technique de numération sélective et miniaturisée, basée sur la consommation spécifique de substrat carboné et la résistance à certains antibiotiques, nous a permis de suivre quantitativement l'évolution de la population de trois genres bactériens (*Lb plantarum*, *Ln mesenteroides* et *Lc lactis*) rencontrés couramment dans les fermentations végétales. Cette technique originale et simple, parfaitement adaptée pour des analyses de routine, a été mise au point avec l'aide de 2 stagiaires (A. Champailler, stage de DEA et Sébastien Moulard, stage d'Ingéniorat INSA). Un article décrivant cette technique a été publié dans la revue Journal of Applied Microbiology (9).

4.2 ETUDE DES MECANISMES SYMBIOTIQUES RHIZOBIUM/ LEGUMINEUSES (1997-2002)

Au cours de ces 8 dernières années, mon intérêt scientifique s'est focalisé de plus en plus sur la compréhension des mécanismes moléculaires, tels que les mécanismes de dégradation de la linamarine ou bien la dégradation de l'amidon brut, les mécanismes évolutifs d'acquisition du caractère amylolytique, initialement par une approche biochimique puis par une approche génétique.

L'opportunité d'orienter mes travaux vers cette voie de recherche plus fondamentale m'a été donnée lors de la création du Laboratoire de Symbioses Tropicales et Méditerranéennes (LSTM). La symbiose Rhizobium/Légumineuses, et en particulier la compréhension des mécanismes symbiotiques entre ces 2 partenaires constitue, en effet, un modèle d'étude particulièrement attrayant et intéressant dans lequel je désirais m'investir.

Le Laboratoire des Symbioses Tropicales et Méditerranéennes (LSTM), auquel je suis rattaché depuis janvier 1997, se propose de développer des recherches fondamentales et appliquées dans les domaines de la biologie, de l'écologie, et de l'utilisation des symbioses plantes-microorganismes dans les zones tropicales et méditerranéennes. Ces recherches portent sur les microorganismes, bactéries fixatrices d'azote et champignons mycorhiziens, qui vivent en symbiose avec des espèces végétales et qui jouent ainsi un rôle important dans l'accroissement ou le maintien durable des productions agricoles et forestières, ainsi que dans la régénération des environnements tropicaux et méditerranéens. Elles permettent ainsi de mettre en place des stratégies de développement durable et de réhabilitation des écosystèmes dégradés et menacés.

Les activités de recherche que j'ai développées depuis mon affectation au LSTM ont visé à caractériser certains microorganismes symbiotiques, étudier leurs propriétés originales, et disséquer certains mécanismes moléculaires.

4.2.1 Caractérisation et propriétés photosynthétiques des *Bradyrhizobium* associés aux *Aeschynomene*

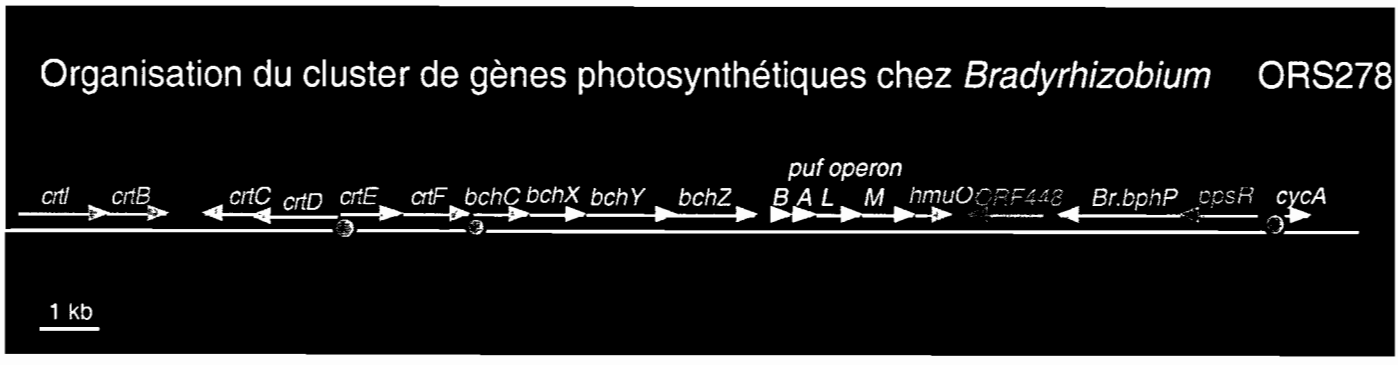
Contrairement à ceux des régions tempérées qui ont été bien étudiés, la plupart des rhizobia des régions tropicales et méditerranéennes sont relativement mal connus. Il est donc indispensable d'acquérir une meilleure connaissance de ces symbioses dont le potentiel reste largement inexploité. Ainsi, certains rhizobia tropicaux ont la propriété, unique parmi tous les rhizobia connus, d'être photosynthétiques. Il s'agit de bactéries du genre *Bradyrhizobium* qui

forment des nodules aériens fixateurs d'azote sur les légumineuses aquatiques appartenant au genre *Aeschynomene* (Evans *et al.* 1990 ; Fleishman & Kramer 1998). De plus cette symbiose est très spécifique. En effet, seuls les *Bradyrhizobium* photosynthétiques nodulent les *Aeschynomene* à nodules de tige (Molouba *et al.* 1999). Les nodules induits par ces rhizobia sur la tige d'*A. sensitiva* sont d'un type nouveau appelé «nodules en collier » car ils sont plats et entourent la tige, et leur morphologie semble permettre de recueillir la lumière de façon optimale. Ces rhizobia appartiennent au genre *Bradyrhizobium*, qui est phylogénétiquement proche des *Rhodospirillaceae*, un groupe de bactéries pourpres photosynthétiques. Cette relation phylogénétique suggère que ces *Bradyrhizobium* ont évolué à partir de bactéries photosynthétiques.

Nos objectifs dans cette étude étaient d'apporter des réponses aux questions suivantes : Quelle est l'origine des gènes photosynthétiques des *Bradyrhizobium* d'*Aeschynomene*? La fonction photosynthétique des *Bradyrhizobium* est-elle exprimée durant la symbiose? Quel est l'impact de cette photosynthèse bactérienne sur le développement des plantes ?

Chez les bactéries photosynthétiques anoxygéniques, l'ensemble des gènes intervenant dans la formation du photosystème sont regroupés dans une région de 45-kb où l'on retrouve les gènes *bch* et *crt* codant respectivement les enzymes de la voie de biosynthèse de la bactériochlorophylle et des caroténoïdes, et les gènes *puf* qui codent les protéines majeures des antennes collectrices et du centre réactionnel (Choudhary & Kaplan, 2000).

Avant notre étude, aucune caractérisation des gènes *puf* n'avait été réalisée chez les *Bradyrhizobium* photosynthétiques. Un criblage d'une banque d'ADN génomique réalisée sur la souche ORS278 (souche modèle du laboratoire) a permis d'isoler deux clones recouvrant une région de l'ordre de 70 kb. Une sous-région de 25 kb regroupant la grande majorité des gènes photosynthétiques (gènes *bch*, *crt* et *puf*) a été entièrement séquencée (voir figure ci-dessous).



Une analyse phylogénétique de la région d'ADN de 1500 pb codant les protéines PufL et PufM a pu être réalisée à partir des séquences disponibles dans GenBank pour toute une série de bactéries photosynthétiques. Il apparaît clairement que les gènes *puf* d'ORS278 sont proches phylogénétiquement de ceux de la bactérie *Rhodospeudomonas palustris*. Ce positionnement phylogénétique se rapproche de celui obtenu à partir de l'analyse de la région du 16S rDNA. Cette relation phylogénétique suggère que ces *Bradyrhizobium* photosynthétiques et *R. palustris* dériveraient d'une même bactérie ancestrale photosynthétique. Leur adaptation à l'environnement particulier des légumineuses à nodules de tige, environnement aquatique favorable au maintien des fonctions photosynthétiques, se serait traduite par la conservation des caractères photosynthétiques conjointement à l'acquisition des caractères symbiotiques spécifiques aux *Aeschynomene* à nodules de tige.

La caractérisation de cet opéron *puf*, nous a permis dans un deuxième temps la construction d'un mutant isogénique non photosynthétique. Ce mutant a été obtenu par une délétion d'un fragment de 800 pb à l'intérieur des gènes *pufL* et *pufM* et l'insertion d'une cassette *lacZ*-Kanamycine. Le gène rapporteur *lacZ*, sous le contrôle de la région promotrice des gènes *puf*, a été utilisé pour étudier l'expression des gènes photosynthétiques durant la symbiose. Les résultats obtenus montrent une expression des gènes bactériens photosynthétiques spécifiquement dans les nodules de tige, ce qui indique que cette photosynthèse bactérienne est fonctionnelle durant la symbiose.

L'obtention du mutant PufLM négatif, nous a permis par ailleurs d'apprécier l'importance de cette photosynthèse bactérienne sur la symbiose. Pour cela, nous avons comparé la croissance des plantes inoculées au niveau des tiges ou des racines avec la souche sauvage ou la souche mutante. Aucune différence significative n'a été observée sur la croissance ou sur la couleur du feuillage des plantes d'*Aeschynomene sensitiva* inoculées au niveau des racines par la souche sauvage ou par la souche mutante. Par contre, lors d'une inoculation au niveau de la tige uniquement, la croissance des plantes mais aussi la nodulation et la fixation de l'azote ont été très sévèrement diminuées (prés de 50 %) avec la souche mutante. Ces observations sont corrélées avec un retard dans l'apparition des premiers nodules et une efficacité de nodulation réduite d'un facteur 2.

Ces résultats, très surprenants par les effets importants observés, montrent un rôle majeur de la photosynthèse bactérienne lors de l'établissement de la symbiose de tige avec *A. sensitiva*.

Cette étude a fait l'objet d'une publication dans la revue PNAS (14) ainsi que d'une conférence dans un congrès international. Par ailleurs, un financement pour ce programme

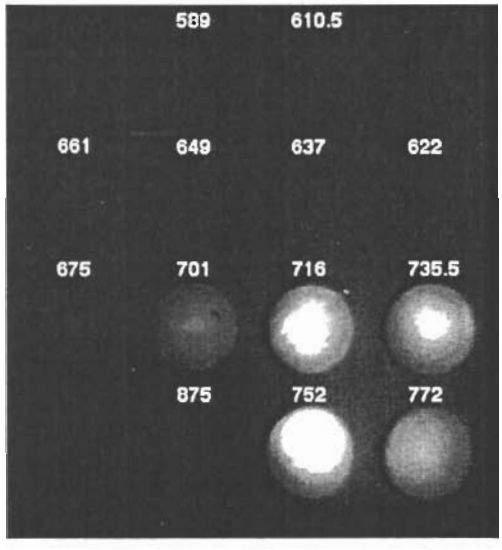
d'étude a été obtenu en 1999 dans le cadre d'un appel d'offre CNRS (Biodiversité et Ecologie des Interactions Durables).

4.2.2 Contrôle de la photosynthèse par un bactériophytochrome.

Le séquençage de la région située en aval de l'opéron *puf* a révélé avec surprise la présence d'une ORF homologue aux phytochromes des plantes. Les phytochromes constituent une famille de capteurs lumineux sensibles au rayonnement rouge et Infra-rouge qui jouent un rôle essentiel dans le développement des plantes (Quail et al. 1995). Ces capteurs de lumière existent sous deux conformations différentes, la forme Pr obtenue après éclairement en lumière Infra-rouge, qui peut être photoconvertie réversiblement en forme Pfr, forme biologiquement active chez les plantes après un éclairement rouge.

La découverte récente de phytochromes chez des bactéries photosynthétiques (Kehoe & Grossman, 1996) et non-photosynthétiques (Davis *et al.* 1999) à soulever l'espoir de pouvoir étudier chez des organismes simples procaryotes, le mode d'action des phytochromes. Cependant si ces (bactério)phytochromes avaient pu être caractérisés au niveau moléculaire et spectral, leurs fonctions restaient jusqu'alors inconnues.

La localisation du gène phytochrome à proximité des gènes photosynthétiques, nous a amenés à penser que celui-ci pouvait intervenir dans le contrôle de la mise en place de l'appareil photosynthétique de la souche ORS278. Pour vérifier une telle hypothèse, nous avons étudié l'impact de la qualité de la lumière sur l'activité photosynthétique, grâce à un système de diode qui permet d'éclairer à des longueurs d'onde bien précises et un système de caméra CCD qui mesure la fluorescence ré-émise par les antennes collectrices du photosystème.



Effet de la qualité de la lumière sur l'activité photosynthétique d'ORS278

Nous avons pu ainsi observer une activité photosynthétique uniquement dans les zones éclairées en proche infra rouge de 700 à 770 nm avec un maximum proche de 750 nm. Le spectre d'action ainsi obtenu se superpose au spectre d'absorption de la forme far-red du phytochrome suggérant ainsi un contrôle de l'activité photosynthétique par ce bactériophytochrome.

Pour apporter une preuve formelle du rôle joué par ce phytochrome, nous avons construit également un mutant dont le gène phytochrome a été délété. Plus aucune activité photosynthétique n'est alors mesurée chez ce mutant quelles que soient les conditions d'éclairement. Ces résultats mettent ainsi en évidence un rôle essentiel du phytochrome dans la mise en place de l'activité photosynthétique chez les *Bradyrhizobium* photosynthétiques. C'est la première démonstration d'un rôle clair d'un phytochrome chez un procaryote.

Ces résultats ont été obtenus grâce à une collaboration avec le Laboratoire de Bioénergétique Cellulaire du CEA de Cadarache sous la responsabilité d'André Verméglio. Une publication commune a été faite dans la revue Nature (18). Nicolas Fourrier, dont j'ai assuré l'encadrement scientifique, a effectué son stage de DEA sur ce projet.

4.2.3 Caractérisation des gènes de biosynthèse de la canthaxanthine chez *Bradyrhizobium* ORS278

L'analyse des pigments produits par les *Bradyrhizobium* photosynthétiques a montré que la souche ORS278 produisait, en plus des pigments photosynthétiques, un pigment supplémentaire la canthaxanthine qui représente plus de 85 % des caroténoïdes totaux produits par la souche (Lorquin *et al.* 1997).

La canthaxanthine est un ß-carotène ayant un fort pouvoir antioxydant pouvant protéger l'organisme contre les dommages photo-oxydatif. Cette molécule, produite par synthèse chimique actuellement, présente une forte valeur ajoutée. En effet, elle est utilisée pour ses propriétés colorantes dans l'industrie agroalimentaire et pour ses propriétés photo-protectrice dans l'industrie cosmétique et pharmaceutique.

La quantité de canthaxanthine produite par la souche ORS278 reste cependant trop faible (1,46 mg/g de MS) pour envisager d'utiliser cette souche comme producteur de canthaxanthine ayant le label "naturel". Cependant, il peut être possible d'augmenter la production de canthaxanthine en clonant et surexprimant les gènes de la voie de biosynthèse de ce caroténoïde.

Nous avons pu isoler ce cluster de gène à partir d'une banque d'ADN génomique de la souche ORS278. Ce cluster a été complètement séquencé. Il contient cinq gènes notés *crtE*,

crtY, crtI, crtB et *crtW* répartis en deux opérons distincts. L'ensemble de ces gènes permet la synthèse de canthaxanthine à partir de Farnesyl pyrophosphate. Par ailleurs, nous avons montré par des études de complémentation que le produit de ces gènes sous contrôle du promoteur *lac*, est fonctionnel dans *E. coli*. L'ensemble de ces résultats ont fait l'objet d'un brevet (20) ainsi que d'une publication dans la revue Journal of Bacteriology (12).

Cette étude dont j'ai assuré la direction scientifique, a été réalisée par Laure Hannibal, technicienne au LSTM, et 2 stagiaires Nicolas Anglès d'Ortoli et Nelly Garcia. Par ailleurs, la société Avantis animal production, a financé une partie de ces recherches, ce qui nous a permis le recrutement d'une Etudiante en stage post-doctoral durant 6 mois (Clémence Chaintreuil).

Le cluster de gène *crt* de la canthaxanthine étant isolé, nous envisageons à présent de le cloner sous un promoteur fort dans un plasmide à large spectre d'hôte et à un grand nombre de copies. Plusieurs types de plasmides et plusieurs promoteurs seront testés. Nous espérons ainsi que la transformation d'ORS278 par ces plasmides permettra d'obtenir une souche sur-exprimant ces gènes et surproduisant ainsi la canthaxanthine. Nous continuerons en parallèle à optimiser le milieu et les conditions de culture pour améliorer la productivité et la production de ce pigment.

4.2.4 Interaction *Bradyrhizobium* photosynthétique/riz

Il avait été observé en 1997 par une équipe Américano-Egyptienne, que la bactérie symbiotique *Rhizobium leguminosarum* bv. *Trifolii* était naturellement présente dans les racines de riz cultivé en rotation avec le trèfle (Yanni *et al.* 1997). Ce travail était ainsi le premier à montrer que les rhizobia pouvaient s'associer à une graminée, et laissait envisager un effet bénéfique apporté sur la croissance du riz.

Au Sénégal, on retrouve dans certaines mares temporaires une espèce de riz sauvage (*Oryza breviligulata*) qui cohabite avec certaines légumineuses aquatiques du genre *Aeschynomene* ou *Sesbania*. Un programme de recherche qui a constitué le travail de thèse de Clémence Chaintreuil, dont j'ai assuré la co-direction avec Bernard Dreyfus, a été ainsi initié afin de déterminer si les symbiontes de ces légumineuses (*Bradyrhizobium* photosynthétiques, *Azorhizobium*) étaient retrouvés associés aux racines de cette espèce de riz sauvage. Par des techniques de piégeage sur plantes et d'identification moléculaire, il a pu être ainsi montré que les racines de riz sauvage prélevées dans cette niche écologique particulière, hébergeaient des *Bradyrhizobium* photosynthétiques identiques aux souches qui nodulent les tiges des *Aeschynomene*. Ainsi, ces rhizobia bénéficient d'une triple niche écologique, une vie libre dans

les eaux et le sol, une vie symbiotique dans les nodules des *Aeschynomene*, et une vie associative et endophytique dans les racines de riz sauvage.

L'inoculation en serre du riz sauvage par différentes souches de *Bradyrhizobium* photosynthétiques, isolés des racines du riz ou des nodules d'*Aeschynomene*, a permis de mettre en évidence une augmentation significative de la croissance du riz inoculé. Plusieurs expériences successives en serre ont permis de sélectionner la souche ORS278 comme la souche la plus efficace. En effet, cette souche a permis d'obtenir en pots une augmentation significative de la croissance aérienne et racinaire du riz et un gain de rendement en grains compris entre 10 à 20%. Ces résultats nous ont encouragés à conduire une expérience aux champs en Guinée Conakry avec l'aide de Mamadou Sow, agronome spécialiste de la culture du riz. La région expérimentale choisie correspond à une rizière de mangrove qui est connue pour donner de faible rendement de production. Les résultats obtenus aux champs sont très encourageants, ils montrent un apport considérable de l'ordre de 25 % sur la croissance du riz et de 15 % sur la production en grains. Au stade actuel de nos connaissances, il devient ainsi indispensable d'intensifier les essais agronomiques, notamment pour sélectionner les combinaisons rhizobium/variétés de riz qui sont les plus efficaces. Par ailleurs, au niveau scientifique, il apparaît très intéressant de comprendre les mécanismes moléculaires qui sont impliqués dans l'établissement de cette association *Brayrhizobium*/riz (voir projet de recherche).

Les résultats obtenus au cours de cette étude ont fait l'objet d'une publication dans la revue Applied and Environmental Microbiology (13).

4.2.5 Caractérisation d'un nouveau groupe de rhizobium methylotrophe associé aux crotalaires

Au Sénégal, on assiste à une dégradation régulière des sols conduisant à la disparition de plusieurs espèces végétales, et causant par conséquent d'importants dommages dans la reconstitution de la biodiversité des écosystèmes forestiers. Cette disparition d'espèces entraîne également une baisse de la production de fourrage et de bois. Il existe certaines espèces de plantes qui contribuent au maintien de la fertilité des sols et à leur enrichissement en matière organique. C'est le cas des légumineuses pérennes qui représentent une partie importante du couvert végétal naturel et 50% d'entre elles présentent des nodules fixateurs d'azote.

Il apparaît donc intéressant d'utiliser les légumineuses en association symbiotique avec les bactéries de la famille des *Rhizobiaceae*, dans les programmes de réhabilitation des sols, notamment dans les zones sèches où elles sont très présentes. Parmi celles-ci, les crotalaires

représentent un grand intérêt car ce sont des légumineuses tropicales qui jouent un rôle considérable dans l'amélioration des sols et en particulier dans les champs mis en jachère.

Dans ce programme de recherche, notre objectif principal a été de caractériser la diversité et les propriétés symbiotiques des rhizobia associés aux espèces du genre *Crotalaria* afin de pouvoir disposer ultérieurement de souches performantes pouvant être employées comme inoculum pour l'utilisation au champ des espèces de crotalaires qui présentent d'importantes potentialités agronomiques.

Une collection de 126 souches de rhizobia isolées à partir des principales espèces de Crotalaires du Sénégal a été ainsi constituée au Laboratoire de Microbiologie IRD de Dakar. Nous avons découvert que plusieurs espèces de Crotalaria (*C. podocarpa, C. glaucoides* et *C. perrottetii*) présentaient une spécificité de nodulation, ne formant des nodules qu'avec des souches à croissance rapide. Leur caractérisation génotypique et phylogénétique montre qu'elles appartiennent au genre *Methylobacterium* et constituent une nouvelle espèce que nous avons nommée "*Methylobacterium nodulans*". L'étude des gènes de nodulation a montré que "*M. nodulans*" est la première espèce de *Methylobacterium* symbiotique connue à ce jour. Elle contient un gène *nodA* proche de celui des *Bradyrhizobium*, suggérant ainsi un transfert latéral de gènes.

La mise en évidence du gène *mxaF* qui code pour la grande sous-unité de la Méthanol deshydrogénase et l'étude de sa fonctionnalité, a démontré que "*M. nodulans*" est méthylotrophe facultatif, caractère unique chez les rhizobia. L'utilisation de mutants spontanés suggère par contre que cette propriété ne jouerait pas un rôle dans l'infection et le développement du nodule dans la symbiose. La découverte des *Methylobacterium* ouvre ainsi d'importantes perspectives dans l'utilisation des légumineuses à des fins écologiques.

Ce travail a été réalisé avec un doctorant, A. Sy dont j'ai assuré avec B. Dreyfus la co-direction. Les résultats obtenus nous ont conduits à écrire deux articles sur ce sujet (16,17) et à déposer un brevet (19). Ce programme de recherche a été repris depuis par Philippe Jourand, Ingénieur d'Etude au LSTM.

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5. PROJET DE RECHERCHE

Mécanismes moléculaires d'adaptation des *Bradyrhizobium* photosynthétiques à leurs environnements.

Le projet de recherche que je me propose de développer s'inscrit dans la poursuite directe des travaux que j'ai menés ces dernières années au LSTM autour des *Bradyrhizobium* d'*Aeschynomene*. Le choix de ce modèle d'étude résulte des propriétés exceptionnelles retrouvées chez ces bactéries i) capacité photosynthétique (propriété unique chez les rhizobia) ii) fixateur libre d'azote (propriété partagée uniquement par *Azorhizobium caulinodans*) iii) environnements multiples (bactérie retrouvée dans le sol ou le milieu aquatique pouvant s'associer symbiotiquement avec les *Aeschynomene* au niveau de nodules racinaires ou caulinaires ou encore colonisant les racines de riz).

Les principaux objectifs scientifiques seront la compréhension de certains mécanismes moléculaires permettant l'adaptation de ces *Bradyrhizobium* photosynthétiques à leurs environnements multiples.

Deux axes de recherche seront plus particulièrement développés :

- La photobiologie de ces bactéries, en étudiant l'effet de la lumière sur la physiologie de ces *Bradyrhizobium* et en s'intéressant plus précisément aux mécanismes de perception et de transduction du signal lumineux capté par le(s) phytochrome(s) identifié(s),

- L'interaction *Bradyrhizobium* photosynthétique/plante, en recherchant les déterminants génétiques bactériens impliqués dans la colonisation de la plante hôte aboutissant soit à une symbiose (*Aeschynomene*) soit à un effet PGPR (Plant Growth Promoting Rhizobia) sur le riz.

5.1 PHOTOBIOLOGIE DES *BRADYRHIZOBIUM* PHOTOSYNTHETIQUES

Avant-propos : cette partie du projet se fera en collaboration étroite avec le Laboratoire de Bioénergétique Cellulaire du CEA de Cadarache sous la responsabilité d'André Verméglio. Nous poursuivrons conjointement les recherches que nous avons menées ces 3 dernières années en travaillant chacun dans nos domaines de spécialisation : i) Biologie moléculaire, Génie génétique et Physiologie au LSTM, ii) Biophysique, Biochimie et Cristallographie au CEA.

De très nombreuses études ont été réalisées ces dernières années pour comprendre les mécanismes de régulation de la mise en place de l'appareil photosynthétique chez les bactéries pourpres (Bauer & Bird, 1996). Pour l'ensemble des bactéries modèles étudiées (*Rhodobacter*

capsulatus, *Rhodobacter sphaeroides*, *Rubrivivax gelatinosus*...), deux paramètres semblent essentiels : i) l'oxygène qui a un effet répresseur important dans la mise en place du photosystème (ce sont des bactéries qui photosynthétisent principalement en anaérobiose) ii) l'intensité lumineuse, qui a plus fable niveau, induit la mise en place des antennes de type LHII. Les travaux que nous avons menés précédemment montraient que les gènes photosynthétiques sont régulés différemment chez les *Bradyrhizobium* d'*Aeschynomene*. En effet ce sont des bactéries aérobies strictes qui mettent en place leur photosystème dans des conditions de microaérophilie uniquement. Par ailleurs, on observe un contrôle total de la mise en place de cet appareil photosynthétique suivant la lumière ambiante, via l'action d'un phytochrome. Nous désirons à présent étudier par quels mécanismes moléculaires ce nouveau phytochrome contrôle l'expression de ces gènes photosynthétiques.

5.1.1 Caractérisation de la chaîne de transduction du signal lumineux

Contexte

Les phytochromes constituent une famille de chromoprotéines formés par l'assemblement de 2 polypeptides identiques sur chacun desquels se fixe de façon auto-catalytique un chromophore de type tetrapyrolle linéaire (biline) (Smith, 2000). Les phytochromes sont ainsi des molécules photosensibles qui changent de conformation suivant la qualité de la lumière. Une barrière majeure de l'étude du fonctionnement de ces phytochromes chez les plantes est liée à la complexité de ces organismes. Pour exemple, chez *Arabidopsis thaliana* qui est l'une des plantes modèles les plus simples, 5 phytochromes (phyA-E) ont été décrits qui semblent interagir avec d'autres récepteurs bleus (les cryptochromes) dans une myriade de combinaisons pour réguler la photomorphogénèse de la plante. La découverte récente de la présence de phytochromes chez les organismes procaryotes a relancé les perspectives d'études. Le modèle simple qu'ils représentent et les possibilités offertes par les outils de génie génétique devraient pouvoir apporter des informations précises sur les mécanismes d'actions chez les phytochromes de plantes (Hughes & Lamparter, 2000). Le premier bactériophytochrome décrit est Cph1 chez *Synechocystis*, une cyanobactérie (Hughes *et al*, 1997). Plusieurs autres ont été découverts chez différentes bactéries photosynthétiques et non photosynthétiques (Davis *et al*, 1999), ce qui a clairement mis en évidence leur présence dans le monde procaryote. Ces bactériophytochromes seraient vraisemblablement les formes ancestrales des phytochromes des plantes supérieures. Ils possèdent en N-terminal un domaine homologue au CBD (Chromophore Binding Domain) des plantes sur lequel peut se fixer un chromophore, et en C-terminal les motifs nécessaires à une activité histidine kinase. Le fait qu'un bactériophytochrome soit une kinase sensible à la lumière a été clairement démontré avec Cph1 dont la première étape de la transmission du signal lumineux met en jeu le transfert d'un

groupement phosphate entre le bactériophytochrome et son partenaire Rcp1 (Yeh *et al,* 1997). Cependant, si cette première étape de transduction du signal lumineux a pu être clairement identifiée, on ne connaît toujours pas quels gènes sont sous le contrôle de ce phytochrome et quel est la suite de la transduction du signal à partir de Rcp1.

Contrairement aux autres bactériophytochromes déjà décrits, l'extrémité C-terminal du phytochrome (Br.bphP) que nous avons caractérisé chez ORS278 ne présente aucune signature histidine-kinase, suggérant ainsi une nouvelle voie de transduction du signal lumineux. Le gène *br.bphP* est contigu à un gène codant un facteur de transcription PpsR. Il a été montré chez des bactéries photosynthétiques pourpres, que PpsR, en fonction du potentiel redox, bloquait l'expression de certains gènes photosynthétiques en se fixant sur une région d'ADN retrouvée en amont de ces gènes (Ponnampalam *et al.* 1995). La synthèse constitutive du photosystème, quelles ques soient les conditions d'éclairement, observé chez un mutant d'ORS278 *ppsr* négatif, suggère que ce facteur de transcription intervient chez les *Bradyrhizobium* photosynthétiques, dans la chaîne de transduction du signal lumineux. Un modèle de régulation de l'activité photosynthétique par le phytochrome et PpsR est proposé. En lumière rouge, le phytochrome serait sous sa forme inactive (Pfr) et n'interagirait pas avec PpsR qui pourrait alors se fixer sur un motif palindromique de l'ADN située en amont des gènes photosynthétiques cibles; un éclairement en lumière infra-rouge photoconvertirait le phytochrome sous la forme active qui pourrait lier PpsR et laisser ainsi libre la transcription des gènes.

Objectifs : ils sont de valider le modèle précédemment proposé en étudiant *in vitro* l'interaction Phytochrome/PpsR/ADN. Dans le cas où un tel modèle ne serait validé, nous rechercherons alors à identifier le(s) partenaire(s) interagissant avec ce phytochrome.

Méthodologies

Interactions *in vitro* Br.BphP/PpsR/ADN

Pour mettre en place une telle étude il est nécessaire de disposer de chacun des partenaires purifiés. Concernant le phytochrome, nous disposons au laboratoire de constructions dans des vecteurs d'expression permettant sa surproduction et sa purification. En ce qui concerne la protéine PpsR, les différents vecteurs testés ont été peu performants et les quantités produites restent trop faibles. Il sera donc nécessaire de tester d'autres vecteurs d'expression en particulier le vecteur pBAD qui a donné récemment les meilleurs résultats pour le phytochrome. Le site de fixation sur l'ADN de PpsR est déjà connu, il a été caractérisé chez les autres bactéries pourpres photosynthétiques (Ponnampalam & Bauer, 1997) et nous avons pu l'identifier chez la souche ORS278 au niveau de la région promotrice des gènes *bchC* et *crtE*. La région ADN correspondante sera tout simplement isolée par PCR.

La mise en évidence d'une interaction entre 2 protéines est toujours une étape délicate et bien souvent plusieurs approches sont nécessaires. Nous utiliserons donc différentes techniques i) par biochimie, en utilisant des agents linkers ou par chromatographie sur gel filtration ii) par biologie moléculaire, grâce à la technique du DNA FootPrint qui permettra d'étudier la fixation de PpsR sur l'ADN et de déterminer si cette fixation est réversible en présence de la forme Pr de Br.BphP.

Recherche d'un autre partenaire interagissant directement avec le phytochrome

Dans le cas où le modèle de régulation à deux composants (Br.BphP/PpsR) ne serait pas vérifié, nous envisageons d'identifier le ou les partenaires manquant dans cette chaîne de transduction du signal lumineux. Nous avons déjà mis en place une banque de mutants au hasard de la souche ORS278 en utilisant un transposon (miniTn5 contenant le gène rapporteur *gus*). Cette banque de plus de 5000 mutants est parfaitement ordonnée en microplaques de 96 puits. Nous avons commencé à cribler cette banque en sélectionnant d'une part les clones qui sont photosynthétiques à l'obscurité et les clones non-photosynthétiques après culture sous 740 nm. Plus d'une cinquantaine de clones ont pu être ainsi sélectionnés et le site d'insertion du Tn5 pour une dizaine d'entre eux vient d'être déterminer. Nous avons pu ainsi identifier des mutants où l'insertion du Tn5 s'est produite dans le gène *ppsR* ou encore dans des gènes de la voie de biosynthèse de la bactériochlorophylle. Ces premiers résultats nous laissent penser que la banque est bien représentative et que cette stratégie nous permettra d'identifier l'ensemble des déterminants génétiques intervenant dans le contrôle de la photosynthèse chez la souche ORS278. Nous poursuivrons donc la caractérisation des mutants ainsi sélectionnés.

Une dernière approche par la technique d'un système d'expression double hybride chez la levure pourra être envisagé si les stratégies précédentes n'aboutissent pas. Il est à noter que c'est par cette approche que l'équipe de Quail (1998, 2002) a pu identifier les facteurs de transcription PIF3 et PIF4 qui interagissent avec le phytochrome phyB chez *Arabidopsis thaliana*.

5.1.2 Vers un système de régulation de gènes d'intérêt par la lumière

Contexte

L'introduction d'un gène chez un organisme vivant (bactérie, plante, animal...) constitue une technique de base à l'origine d'une multitude d'applications dans le domaine de la biologie. Dans de nombreux cas, un système constitutif peut être suffisant. Cependant, dans des cas beaucoup plus complexes, en particulier en recherche et en thérapie génique, l'effet bénéfique que l'on peut espérer de l'introduction d'un nouveau gène va dépendre essentiellement du contrôle que l'on va pouvoir exercer sur son expression. Il est alors capital de pouvoir moduler et contrôler précisément la quantité délivrée du produit de ces gènes.

A ces fins, plusieurs recherches ont été conduites ces dernières années afin de développer de nouveaux outils permettant d'induire et de contrôler l'expression d'un gène. Le système le plus couramment utilisé aujourd'hui est un système d'induction par les sucres tel que l'opéron Lac. Le niveau d'expression du gène peut alors être contrôlé par la concentration en sucre inducteur ou par une molécule similaire telle que l'IPTG. On peut également citer des systèmes d'induction par la température où le gène d'intérêt est induit pour des températures proches de 40°C, des systèmes d'induction par le pH, où le gène est contrôlé suivant l'acidité du milieu, et des systèmes beaucoup plus complexes où l'induction du gène est contrôlée par des drogues (tétracycline, ecdysone, antiprogestine...). L'ensemble de ces systèmes implique l'introduction dans l'organisme d'un ou plusieurs gènes codant pour une ou plusieurs protéines qui vont jouer le rôle de capteurs (capteur de pH, de température, de drogue...) et qui, en fonction des informations perçues vont induire ou réprimer un gène d'intérêt. La plupart de ces systèmes sont peu souples et difficilement réversibles. Il est en effet bien souvent impossible de retrouver un état non-induit en raison, par exemple, de la difficulté de supprimer un agent chimique inducteur. Par ailleurs, ce sont des systèmes qui ne sont pas dépourvus d'innocuité pour l'organisme, en particulier, ceux utilisant des drogues qui ont souvent de nombreux effets secondaires.

Un système de contrôle des gènes par la lumière *via* un phytochrome présenterait de formidables avantages par rapport aux systèmes existants. En effet, Les phytochromes agissent comme de véritables interrupteurs lumineux, il est donc très facile de passer d'un état induit à un état répressif, et vice-versa, de l'expression du gène souhaité, tout simplement en commutant l'éclairement. Par ailleurs, la lumière est un paramètre que l'on sait parfaitement maîtriser, il existe à l'heure différents systèmes (lampes filtrées, diodes électroluminescentes, laser,...) qui permettent d'avoir un éclairement à la longueur d'onde souhaitée. En ce qui concerne le changement d'éclairement et le contrôle du temps d'exposition, un dispositif électronique très simple est suffisant. De plus, le niveau énergétique de la lumière nécessaire à l'induction ou à la répression est suffisamment faible pour induire aucun effet secondaire.

Objectif : Transposer la chaîne de régulation par la lumière *via* le phytochrome et PpSR identifié chez ORS278 pour la régulation de gène d'intérêt dans différents organismes.

Méthodologie : Pour qu'un tel système de régulation fonctionne chez un autre organisme, il faudra insérer chez celui-ci le gène *br.bphp* qui code la partie protéique du phytochrome, le gène *hmuO* qui permet la synthèse du chromophore de ce phytochrome, le gène *ppsR* et éventuellement le(s) autre(s) déterminant(s) génétique(s) intervenant dans la chaîne de

régulation. Ces gènes s'expriment de façon constitutive chez la souche ORS278 et l'on peut supposer qu'il en est de même chez de nombreux autres organismes. Cependant, dans certains cas, ils devront être clonés en aval d'un promoteur constitutif propre à l'organisme que l'on veut modifier génétiquement. Il est à noter que s'agissant de protéines régulatrices, elles agissent probablement à de très faibles quantités, il n'est donc pas nécessaire d'atteindre un niveau d'expression élevé de ces différents gènes. En ce qui concerne le gène d'intérêt dont on voudrait réguler l'expression, celui-ci serait quant à lui intégré en aval de la région promotrice sur laquelle se fixe la protéine PpsR, région dont on connaît la séquence. Une stratégie propre pour chaque organisme est à mettre en place, mais nous pensons que nous disposons à l'heure actuelle de l'ensemble des outils moléculaires pour introduire ce système dans les principaux organismes modèles étudiés (*E. coli*, et levure...).

5.1.3 Cristallisation du phytochrome

Contexte

L'un des principaux challenge en photobiologie est la compréhension des changements de conformation du phytochrome induits lors de la perception du signal lumineux ainsi que les mécanismes de transduction du signal. Pour cela, la cristallisation d'un phytochrome semble être la meilleure approche. L'obtention en 1997, d'un phytochrome recombinant de la cyanobactérie *Synechocystis* a relancé l'espoir d'obtenir en quantité suffisamment pure ce photorécepteur pour envisager sa cristallisation (Lamparter *et al.* 1997). Cependant, malgré les quantités considérables de matériel obtenu (de l'ordre de plusieurs dizaines de milligramme par litre de culture) aucune équipe de recherche n'a réussi à ce jour à cristalliser un phytochrome. Le phytochrome d'ORS278 par rapport à ceux précédemment étudiés présente 2 différences majeures : i) le chromophore, qui est la biliverdine (tétrapyrolle linéaire le plus simple directement synthétisé par l'action d'une hème oxygénase) ii) l'absence d'un domaine Histidine kinase en C-terminal responsable de l'autophosphorylation du phytochrome et du transfert du résidu phosphate au régulateur.

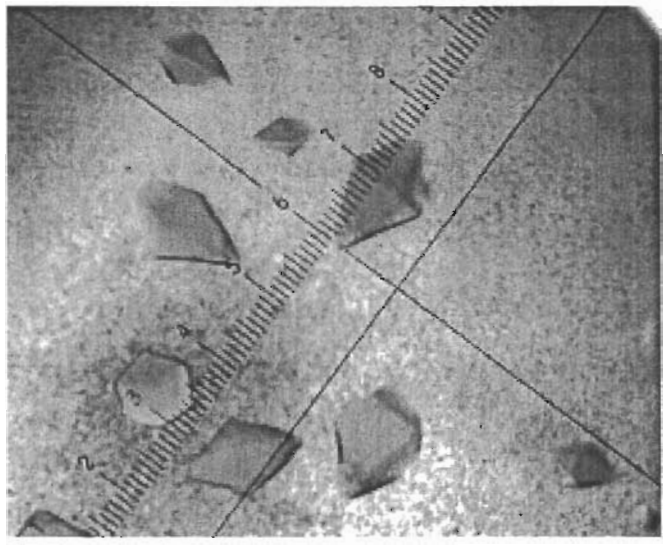
Ces deux caractéristiques pourraient faciliter l'étape de cristallisation de ce nouveau phytochrome isolé de la souche ORS278.

Objectif : cristalliser les différentes conformations d'un phytochrome afin de comprendre comment celui-ci fonctionne.

Méthodologie.

Dans ce travail de collaboration entre le CEA et le LSTM, je suis chargé d'optimiser les constructions permettant de surproduire et purifier un phytochrome recombinant fonctionnel. Pour cela un premier essai a été réalisé au cours duquel nous avons cloné dans un même vecteur d'expression (pBAD), le gène codant l'apophytochrome et celui de la hème oxygènase catalysant la synthèse du chromophore. Ces travaux viennent d'être réalisés par Marianne Jaubert durant son stage de Maîtrise effectué sous ma direction. Par cette approche, il a pu être obtenu jusqu'à 20 mg/l de phytochrome quasiment pur, et parfaitement fonctionnel. Les premiers essais de cristallisation conduits par David Pignol au CEA de Cadarache sont très prometteurs et ont déjà permis d'obtenir des premiers objets cristallins (voir photo ci dessous). Nous envisageons également par cette même approche cristalliser la protéine PpsR et éventuellement de co-cristalliser le phytochrome et son éventuel partenaire PpsR. Il faut noter que ces essais de cristallisation sont effectués soit à l'obscurité, soit sous éclairement continu à une longueur d'onde de 750 nm afin d'assurer une homogénéité de conformation des différentes molécules de phytochromes. Outre ces expériences de cristallisation qui s'avèrent souvent longues et laborieuses, de nombreuses études structurales sont envisageables pour caractériser au niveau moléculaire ce système de réponse à la lumière :

- Les changements de conformation entre les formes Pr et Pfr pourront être étudiés d'un point de vue global, par dichroïsme circulaire ou FTIR
- Les interactions phytochrome-PpsR pourront être étudiées par la technique de diffusion des neutrons avec variation de phase.



Premiers cristaux obtenus avec le phytochrome Br.bphP

5.1.4 Identification d'autres fonctions régulatrices exercées par la lumière.

Contexte : Nous avons pu identifier, par interrogation des banques de données, un gène homologue au phytochrome d'ORS278 chez *Rhodopseudomonas palustris*, espèce très proche phylogénétiquement des *Bradyrhizobium* et dont le génome vient d'être entièrement séquencé. Ce gène noté *rp.bphP* est retrouvé, tout comme celui d'ORS278, à proximité du cluster de gènes photosynthétiques et contigu au gène *ppsR*. De façon identique à la souche ORS278, nous avons observé que la mise en place du photosystème chez *R. palustris* est régulée suivant la qualité de la lumière. Ceci suggère, également chez cette bactérie, une régulation de l'activité photosynthétique *via* le phytochrome Rp.bphP. L'analyse de la séquence du génome de *R. palustris* révèle avec surprise la présence de 5 autres gènes codant aussi pour un phytochrome. Ces différents gènes sont répartis sur le génome et présentent des différences structurales au niveau de l'extrémité C-terminal. Cela nous conduit à penser que d'autres fonctions cellulaires chez cette bactérie sont sous l'influence de la lumière grâce aux contrôles de ces autres phytochromes.

En accord avec cette hypothèse, des observations préliminaires réalisées sur le *Bradyrhizobium* photosynthétique ORS278 et *R. palustris* nous ont permis de mettre en évidence une diminution de l'activité respiratoire ainsi qu'une baisse de croissance dans les zones éclairées sous 740nm. Nous supposons ainsi qu'une co-régulation de la mise en place de la chaîne respiratoire et de l'appareil photosynthétique par la lumière, suivant l'action d'un ou plusieurs phytochromes, pourrait exister chez ces bactéries. Nous avançons l'hypothèse selon laquelle le(s) phytochrome(s) joueraient un rôle central, comme cela a déjà été observé pour RegA/RegB (Joshi & Tabita), dans le contrôle de plusieurs fonctions clefs (photosynthèse, respiration, fixation d'azote...).

Objectifs : Définir les fonctions cellulaires sous le contrôle de la lumière et le rôle des phytochromes.

Méthodologie.

Afin d'avoir une vision globale de l'influence de la lumière sur la physiologie de la bactérie, nous envisageons d'utiliser une approche de type protéomique. On utilisera dans un premier temps comme bactérie modèle, *R. palustris,* dont la séquence complète du génome est connue. Des cultures seront réalisées sous différentes conditions d'éclairement (obscurité, lumière bleue, lumière rouge, lumière Infrarouge et lumière blanche). On comparera pour ces différents essais, les profils protéiques en gel 2D des différents compartiments cellulaires (protéines cytoplasmiques, protéines périplasmiques et protéines membranaires). Les spots les plus significatifs pour une condition donnée seront analysés par MALDI-TOF mass

spectrométrie afin d'identifier précisément la protéine correspondante par comparaison avec la banque de donnée disponible.

Par ailleurs des mutants isogéniques de chacun des 6 gènes de phytochrome seront réalisés afin de préciser leur rôle individuel.

Ce programme, très ambitieux, se fera grâce à une collaboration entre le LSTM, le CEA de Cadarache et le CEA de Grenoble. Le LSTM se chargera des cultures dans les différentes conditions d'éclairement et la construction des différents mutants, le Laboratoire de Bioénergétique Cellulaire du CEA de Cadarache réalisera la préparation et la séparation en gel bi-dimensionnelle des différents extraits protéiques, le Laboratoire de Chimie des Protéines du CEA de Grenoble se chargera de l'analyse des différents spots.

En ce qui concerne la souche ORS278, à défaut de disposer pour l'instant de la séquence complète du génome, on utilisera une approche beaucoup plus ciblée en étudiant plus particulièrement l'effet de la lumière sur la mise en place de la chaîne respiratoire et plus spécifiquement, le niveau d'expression de la cytochrome oxydase et l'ubiquinol oxydase. De plus, nous chercherons, par des techniques de PCR et d'hybridation, à identifier d'autres gènes de phytochromes et préciser alors leurs fonctions.

5.2 INTERACTION *BRADYRHIZOBIUM* PHOTOSYNTHETIQUE/PLANTE

Avant propos : Le développement de ce programme de recherche dépend de la venue prochaine de Frédérique Ampe (Chercheur de l'IRD).

Ce programme a pour objectif de répondre à un certain nombre de questions fondamentales dont les principales sont les suivantes :

Quels sont les mécanismes moléculaires qui sont impliqués au cours de l'interaction symbiotique ou mutualiste entre la bactérie modèle et la plante ?

Quels sont les gènes bactériens majeurs qui sont impliqués durant la colonisation de la rhizosphère d'une plante ?

Comment la plante peut-elle influencer la physiologie de la bactérie ?

La bactérie modèle de cette étude sera le *Bradyrhizobium* photosynthétique ORS278. Deux modèles d'interaction seront étudiés : une interaction de type symbiotique (*Bradyrhizobium/ Aeschynomene*), une interaction de type PGPR (*Bradyrhizobium* /riz)

5.2.1 *Bradyrhizobium/Aeschynomene*

Au cours de nos travaux précédents, nous avons pu mettre en évidence un rôle essentiel de la photosynthèse bactérienne dans la symbiose de tige. Cependant un certain nombre d'interrogation demeurent :

Quels sont les déterminants génétiques responsables de la nodulation ? En effet, quelle que soit la stratégie utilisée (PCR, hybridation, complémentation...), nous avons jusqu'à présent échoué dans l'isolement des gènes de nodulation de la souche ORS278 et des autres *Bradyrhizobium* photosynthétiques appartenant à ce même groupe (voir article 15). Ce qui suggère soit des gènes *nod* très différents de ceux jusqu'à présent décrits, soit un nouveau mécanisme moléculaire impliqué dans la nodulation (il est à noter à ce sujet que les nodules de tige sur *Aeschynomene* ont une morphologie unique). Pour identifier ce(s) déterminant(s) génétique(s), nous envisageons d'utiliser une approche plus contraignante qui consistera à cribler la banque de mutant sur plante et à sélectionner les clones ne conduisant pas à la formation de nodules. Nous caractériserons alors le site d'insertion du Tn5 pour ces différents clones pour identifier le(s) déterminant(s) génétique(s) impliqué(s) lors de cette association.

Quel est le rôle du phytochrome au cours de la symbiose ? Comme nous l'avons vu précédemment, nous supposons que les phytochromes jouent un rôle central sur le métabolisme de la bactérie. *In planta*, nous avons pu montrer que le phytochrome bactérien va permettre la mise en place de l'appareil photosynthétique spécifiquement dans le nodule de tige. Cependant, on peut supposer que ce phytochrome est très probablement impliqué dans le contrôle de l'activité respiratoire de la bactérie et régule ainsi le flux énergétique provenant soit de l'activité photosynthétique soit de l'activité respiratoire, grâce à l'oxydation des substrats carbonés fournis par la plante. Pour apporter des éléments de réponse, nous étudierons le phénotype d'un mutant phytochrome négatif au cours de la symbiose de tige ou de racine. Nous étudierons par ailleurs l'influence de la lumière Infra-rouge sur la nodulation racinaire en éclairant les racines de plantes cultivées en tube.

Pour avoir enfin une vision globale des différences de comportement de la bactérie cultivée sous forme libre ou associée à la plante dans un nodule de tige ou de racine, nous envisageons, grâce à la venue de Frédérique Ampe, utiliser une approche génomique par une analyse de type transcriptome. Ne disposant pas pour l'instant de la séquence du génome, nous utiliserons une approche en aveugle qui consistera à la construction d'une banque de clones avec des inserts de 0.5-1.5 kb couvrant l'ensemble du génome. Nous fabriquerons alors des membranes avec l'ensemble de ces clones (env.10000) et, par hybridation avec des extraits d'ARN marqués obtenus après croissance de la bactérie dans différentes conditions, nous

identifierons les gènes spécifiquement induits. Les clones ainsi identifiés seront séquencés afin de caractériser les gènes impliqués dans l'adaptation de la bactérie à un environnement donné. Dans certains cas, il sera également possible de séquencer les régions flanquant le gène identifié grâce à la banque de cosmides (inserts 30-40 kb) dont nous disposons. Il est à noter qu'une telle approche en aveugle vient d'être utilisée avec succès pour identifier les gènes d'*Erwinia chrysanthemi* spécifiquement induits ou réprimés lors de l'infection de la plante hôte (Okinaka *et al.* 2002).

Les gènes identifiés, spécifiques de l'adaptation de la bactérie à un environnement donné, pourront alors être mutés pour confirmer leur(s) fonction(s).

Il est à noter que cette approche génomique pourra être également utilisée pour la recherche des gènes sous le contrôle de la lumière chez les *Bradyrhizobium* photosynthétiques.

5.2.2 *Bradyrhizobium*/riz

Les premiers essais d'inoculation en rizière avec des *Bradyrhizobium* photosynthétiques sont très prometteurs, et représentent un potentiel certain pour le développement. Ils méritent ainsi d'être transférés à plus grande échelle sur le terrain en multipliant les essais agronomiques avec certains de nos partenaires des pays du sud (Egypte, Inde, Côte d'Ivoire) et en France avec le Centre Français du Riz. Nous souhaiterions, concernant ce dernier point, qu'un Ingénieur Agronome rejoigne notre équipe pour renforcer ces essais. Sur le plan plus académique, il nous paraît indispensable d'étudier les mécanismes moléculaires intervenant entre la bactérie et le riz afin d'améliorer à terme l'efficacité d'une telle association.

L'objectif principal de ce volet fondamental sera donc de mettre en évidence des gènes bactériens impliqués dans l'association bénéfique de la souche ORS278 avec le riz. La première étape consistera à mettre au point un test biologique *in vitro* qui soit simple, pratique et permettant de mettre en évidence des différences significatives entre les plants inoculés ou non.

Nous avons déjà testé de façon préliminaire différents systèmes de culture sur le riz et sur *Arabidopsis thaliana*. Il apparaît que l'effet inducteur de l'élongation des racines n'est pas spécifique du riz et qu'*Arabidopsis* y est également sensible. La croissance de la racine du riz étant très rapide, nous développerons sur le riz un système précoce permettant de mesurer la croissance des racines de plantules âgées d'une semaine et nous mettrons au point pour les grandes séries de tests, un système de mesure du gain de croissance d'*Arabidopsis*.

En disposant d'un tel test biologique, nous pourrons alors rechercher les gènes impliqués dans la croissance racinaire du riz par criblage de la banque de 5000 mutants mini*Tn5* de la souche ORS278. Après avoir sélectionné les mutants n'induisant plus d'effet sur la croissance

du riz, nous séquencerons la région d'insertion du *Tn5* afin de déterminer la séquence de gènes bactériens impliqués.

La mise au point d'un test *in vitro*, tel que nous l'envisageons pour le criblage de plusieurs milliers de mutants, risque de constituer l'un des principal obstacle dans la réussite de ce projet. Aussi nous envisageons également d'étudier les effets de l'inoculation de *Rhodopseudomonas palustris* sur la croissance du riz. Ces bactéries qui sont apparentées aux *Bradyrhizobium* photosynthétiques entraînent également une augmentation importante de la croissance du système racinaire d'*Arabidopsis thaliana* (observation préliminaire réalisée au LSTM). Si des effets positifs sont également observés sur la croissance du riz, nous pourrons utiliser une approche plus ciblée en recherchant sur le génome de la bactérie les gènes susceptibles d'intervenir dans cette augmentation de croissance de la plante (gène *nif*, gènes de la voie de biosynthèse des phytohormones de type auxine ou gibbérelline, gènes pouvant être impliqués dans le transport du N, P K et Fe ...). Les principaux gènes candidats seront mutés et l'on étudiera l'effet de leur inactivation sur l'interaction avec la plante.

D'autre part, ce projet devrait faciliter les collaborations avec les autres UMR de l'IRD centrées autour du riz, et en particulier l'UMR Génomique du riz de Michel Delseny et Alain Ghesquière. Cette collaboration devrait en effet s'élargir à moyen terme vers l'utilisation des puces à ADN de riz qui seront prochainement disponibles grâce à Génoplante, et qui permettront d'identifier les gènes du riz induits par la bactérie. Dans le cas d'une interaction positive *R. palustris* /riz, nous aurions la chance d'avoir un modèle unique dans lequel le génome des 2 partenaires est entièrement caractérisé .

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ARTICLE 3

Giraud E, Brauman A, Keleke S, Lelong B et Raimbault M.

Isolation and physiological study of an amylolytic strain of *Lactobacillus plantarum*

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Applied and Microbiology Biotechnology

Isolation and physiological study of an amylolytic strain of *Lactobacillus plantarum*

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Summary. An amylolytic lactic acid bacterium identified as *Lactobacillus plantarum* was isolated from cassava roots (*Manihot esculenta* var. Ngansa) during retting. The amylolytic enzyme synthesized was an extracellular α-amylase with an optimum pH of 5.0 and an optimum temperature of 55° C. Cultured on starch, the strain displayed a growth rate of 0.43 h^{-1}, a biomass yield of 0.19 $g \cdot g^{-1}$ and a lactate yield of 0.81 $g \cdot g^{-1}$. The growth kinetics were similar on starch and glucose. Sufficient enzyme was synthesized and starch hydrolysis was not a limiting factor for growth. Biosynthesis of the enzyme was observed when the glucose concentration was less than 6.7 $g \cdot l^{-1}$ and reached up to 4 $IU \cdot ml^{-1}$ at the end of the fermentation.

Introduction

The lactic microflora plays an important role in the preparation of traditional foods based on fermented cassava (gari, chikwangue, foo-foo) (Regez et al. 1988; Okafor et al. 1984). However, the function of this microflora in the preservation of foods, the detoxification of cyanide compounds and the improvement of organoleptic qualities has not yet been perfectly elucidated. Artisanal methods are still used for manufacturing these foods (Muchnik and Vinck 1984). As the fermentation stage occurs naturally with epiphytic lactic microflora, the quality of the food products is not very well standardized. The mass inoculation of cassava roots with one or several selected strains would enable better control of natural fermentation and to orientate it towards the production of a better-quality, standardized product, hence the interest of better knowledge of the nature and function of this epiphytic microflora.

As cassava consists mainly of starch (over 80% dry matter; Ketiku and Oyenuga 1970), the selection of a lactic acid bacterium capable of metabolizing starch appears to be particularly important. It should be noted that the capacity of lactic acid bacteria to convert starch into lactic acid is not a frequent characteristics. Only species of *Streptococcus bovis, S. equinus, Lactobacillus amylophilus, L. amylovorus, L. acidophilus, L. cellobiosus,* (and others isolated from animal digestive tracts and plant wastes) have been described in the literature as amylolytic lactic acid bacteria (Cotta 1988; Champ et al. 1983; Nakaruma and Crowell 1979; Nakaruma 1981; Sen and Chakrabarty 1986; Sneath 1986). There is practically no information on the physiology of these microorganisms.

The present article describes the isolation and identificaton of a new amylolytic lactic acid bacterium from cassava roots during retting. The physiology of the bacterium and the properties of the amylase produced were also investigated.

Materials and methods

Origin of the plant material. Cassava roots (*Manihot esculenta* var. Ngansa) were harvested in the Brazzaville area 15 months after planting.

Isolation and identification of strains. Peeled roots were immersed in rain-water. Sampling was carried out 4 days after fermentation by random selection of six roots cut into 0.5-cm cubes and mixed under sterile conditions. Sixty grams was sampled and diluted in 540 ml sterile peptone solution. Decimal dilutions (0.1 ml) were spread on JP2 medium (see below) in Petri dishes. After incubation for 48 h at 30° C, dishes were exposed to iodine vapour to detect starch hydrolysis areas. Strains isolated were then purified by three successive transfers on JP2 medium and cultures were routinely checked for purity by microscopic observation. Microorganism identification was based on the following examinations: (1) configuration of the lactic acid produced using an enzymatic method (Ivorec-Szylit and Szylit 1965) with dehydrogenase L and D (Boehringer, Mannheim, FRG), (2) homolactic or heterolactic character, determined by acetic acid or ethanol production; (3) absence of catalase, (4) microscopic and macroscopic examination of morphology, mobility and spores, (5) Gram stain, (6) arginine deamination, (7) growth at 15° and 45° C, (8) fermentation of different carbon sources (API 50CH no. 5030 strips, Biomérieux, Charbonnieres les Bains, France). Evaluation of results and

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identification of the different strains were carried out using Bergey's Manual (Sneath 1986).

Strains and culture media. Three strains were used as reference: *L. plantarum* (Lacto Labo, Dange, St. Romain, France), *S. equinus* CNCM 103233 and *L. amylophilus* CNCM 102988T.

The JP2 medium contained ($g \cdot l^{-1}$): M66 universal peptone, 2.5; soya peptone obtained by papain digestion, 5; casein peptone obtained by pancreatic digestion, 2.5; yeast extract, 5; meat extract, 2.5; $MgSO_4 \cdot 7H_2O$, 0.1; NaCl, 3; $(NH_4)_2SO_4$, 2; K_2HPO_4, 0.2; Prolabo (Paris, France) soluble starch, 3; Tween 80, 0.4 ml. The pH was adjusted to 6.75 before sterilisation.

Physiological studies were performed using an MRS basal medium (de Man et al. 1960) by changing the carbon sources as follows: (a) 5% glucose, (b) 5% starch and (c) 3% of starch plus 2% glucose.

Culture conditions. Strains were cultured in a 2-l bioreactor (Biolafitte, Poissy, France) at 30° C and agitated at 200 rpm. The pH was adjusted to 6.0 by the addition of NaOH 5 N. Inoculation at 10% v/v was performed with a 20-h pre-culture in the same medium used for fermentation.

Analytical methods. The biomass concentration was determined by measurement of optical density (OD) at 540 nm related to the dry weight measured after two washing and centrifugation cycles and drying at 105° C for 24 h. For starch cultures, hydrolysis of residual starch was performed using a mixture of amylases (thermamyl + dextrosyme supplied by Novo). The dry weight and OD were then determined as above. Lactic acid, glucose, acetic acid and ethanol concentrations in the supernatant were assayed by HPLC. Compounds were separated by using an Aminex HPX 87H column (BioRad, Richmond, Va., USA) with a 0.8 $ml \cdot min^{-1}$ flow (pump LDC 3200) of 0.006 M H_2SO_4 at 65° C. Analyses were carried out by a refractive index detector (PU 4026 Philips, Eindhoven). Total sugars in media containing starch were also determined with anthrone using the method of Dubois et al. (1956).

Determination of amylase activity. Enzymatic extracts were prepared by ultrafiltration of culture supernatant using a Diaflo PM 10 membrane (Amicon, Danvers, USA). Enzymatic activities were then assayed by adding 0.1 ml enzymatic extract to 0.8 ml of a solution containing 1.2% of soluble starch (Prolabo) in 0.1 M phosphate buffer, pH 6.0. The reaction was stopped by addition of 0.1 ml of 5 M NaOH. After incubation for 10 min at 40° C, the increase in reducing power was determined using the method of Miller (1959). One enzyme unit is defined as the amount of enzyme that releases one micromole of reducing power equivalent per minute under the conditions described.

Characteristics of the enzyme. The effect of pH on the enzyme activity was studied in a 2.5-8.0 pH range using a 0.1 M citrate-phosphate or 0.1 M TRIS-HCL buffer. Enzymatic activity was measured under standard conditions (see above), except for variation of the buffer. The enzymatic activity profile according to temperature was determined within a 10-80° C temperature range under standard conditions.

Results

Isolation and identification of L. plantarum A6

Seven amylolytic microorganisms were isolated on JP2 medium from retted cassava roots. Two of them were selected for their capacity to produce lactic acid from starch (revealed by HPLC). Morphological, physiological and biochemical characteristics of these two microorganisms are presented Table 1. The ability of these cultures to utilize 49 different carbohydrates was studied using API 50CH no. 5030 strips. The results were compared, using a computer, with the percentage positive reactions of different *Lactobacillus* spp. as per API: 99.9% of similarity with *L. plantarum* was observed and hence these cultures were identified as strains of *L. plantarum*. The two strains A6 and A43, which displayed precisely the same sugar degradation profiles, are probably similar.

The amylolytic activities on JP2 medium of *L. plantarum* A6, *S. equinus* and *L. amylophilus* indicated that the starch hydrolysis zone was largest for *L. plantarum* A6. It was therefore selected for further studies.

L. plantarum A6 growth kinetics

Figures 1, 2 and 3, represent growth kinetics, sugar consumption, lactic acid and amylolytic enzyme production by *L. plantarum* A6 cultured on different media as indicated in Materials and methods. The main fermentation parameters are shown in Table 2. It was observed (Table 2) that the growth of *L. plantarum* A6 on glucose MRS medium was fully comparable with that of *L. plantarum* (Lacto Labo). The growth rate ($0.43\ h^{-1}$) and biomass productivity ($0.75\ g \cdot l^{-1} \cdot h^{-1}$) were slightly lower than those of the standard (Lacto Labo) strain, but the biomass and lactate yields were practically identical. The strain thus does not seem to display specific nutritional requirements in comparison to the

Table 1. The characteristic of *Lactobacillus plantarum* strains A6, A43 and Lacto Labo

Strain	A6	A43	Lacto labo
Ratio of D/L-lactic acid	(69/31)	(66/34)	(73/27)
Homolactic	+	+	+
Catalase	−	−	−
Bacterium shape	Short rod	Short rod	Short rod
Gram stain	+	+	+
Spore	−	−	−
Mobility	−	−	−
Deamination of arginine	−	−	−
Growth at 15° C	+	+	+
Growth at 45° C	−	−	−

+, positive; −, negative

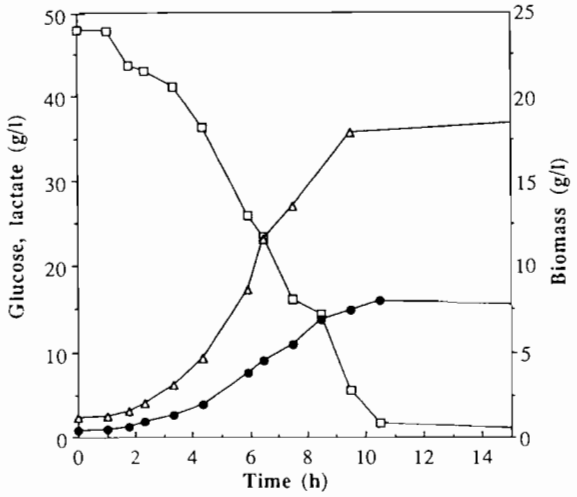

Fig. 1. Fermentation of *Lactobacillus plantarum* A6 on glucose MRS medium at 30° C and pH 6.0: □, glucose; △, lactic acid; ●, biomass

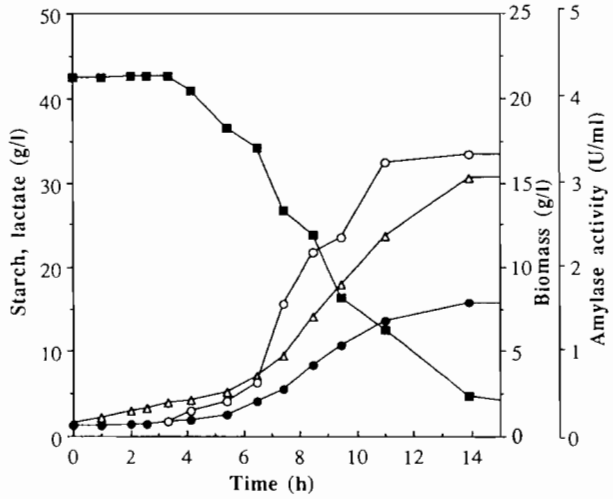

Fig. 2. Fermentation of *L. plantarum* A6 on starch MRS medium at 30° C and pH 6.0: ■, starch; △, lactic acid; ●, biomass; ○, amylase activity

common strain, which makes it possible to envisage mass production.

On starch MRS medium (Fig. 2), the strain exhibited the same kinetic profiles and the same yields. A level of 3.3 IU·ml^{-1} of enzyme had been synthesized at the end of fermentation; nearly 50% was synthesized during the

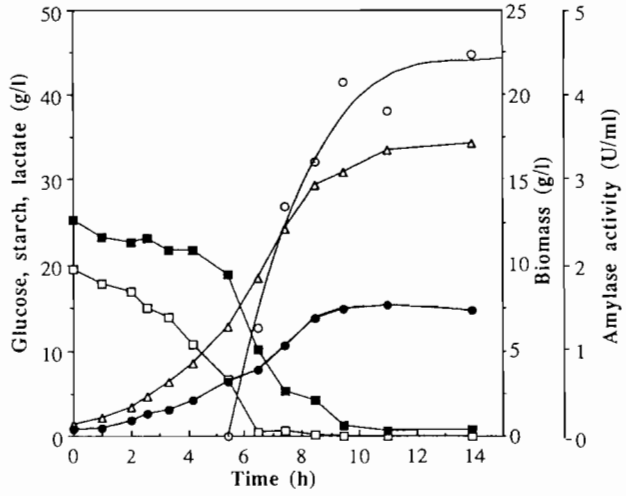

Fig. 3. Fermentation of *L. plantarum* A6 on starch + glucose MRS medium at 30° C and pH 6.0: □, glucose; ■, starch; △, lactic acid; ●, biomass; ○, amylase activity

decreasing phase of growth. The rate of starch hydrolysis was greater than the uptake rate, leading to a 3 g·l^{-1} maltose peak during the 7th hour of fermentation (result not shown). Hydrolysis of starch is thus not a limiting factor.

The kinetics on starch + glucose MRS medium (Fig. 3) indicate that the rate of starch breakdown is very low and amounted to about 14% at the end of 4 h fermentation. Also, no α-amylase was formed up to this stage, thereby indicating that the enzyme present in the inoculum was responsible for the starch breakdown up to 4 h of the fermentation. It thus appears that α-amylase was not formed as long as the glucose concentration remained at about 6.7 g·l^{-1}. Extremely rapid uptake of starch was then observed and it correlated well with considerable synthesis of amylase. Growth kinetics showed a constant rate and rapid adaptation to change of substrate. Amylase concentration at the end of the fermentation (4.3 IU·ml^{-1}) was distinctly higher than that observed on starch MRS medium.

The residual starch concentrations at 14 h in starch MRS and starch + glucose MRS media were different; 4.71 g·l^{-1} in the former as against 0.9 g·l^{-1} in the latter medium. These difference are probably related to initial starch concentration in the media and the presence of readily utilizable glucose in the latter medium. The rate of biomass formation was comparatively slow in starch MRS medium in the initial stages of fermenta-

Table 2. Fermentation parameters of *L. plantarum* (Lacto Labo) and *L. plantarum* A6 cultured on different media at pH 6.0 and 30° C

Strain	Medium	μ (h^{-1})	$Y_{x/s}$ ($g \cdot g^{-1}$)	$Y_{p/s}$ ($g \cdot g^{-1}$)	Productivity ($g \cdot l^{-1} \cdot h^{-1}$)
L. plantarum (Lactolabo)	MRS (glucose, 50 g/l)	0.57 ± 0.03	0.22 ± 0.03	0.75 ± 0.04	1.05
L. plantarum A6	MRS (glucose, 50g/l)	0.43 ± 0.03	0.19 ± 0.02	0.81 ± 0.10	0.78
L. plantarum A6	MRS (starch, 50 g/l)	0.45 ± 0.03	0.23 ± 0.03	0.75 ± 0.10	0.63
L. plantarum A6	MRS (starch 30 g/l + glucose 20 g/l)	0.41 ± 0.06	0.19 ± 0.02	0.74 ± 0.06	0.71

tion. For example, the biomass formation at 4 h was 0.4 and 2 $g \cdot l^{-1}$ in starch MRS and starch + glucose MRS media, respectively. The α-amylase formation was initiated at 2.5 h in starch MRS medium and its rate of formation was very low up to 6 h. In contrast, the enzyme was not detectable up to 6 h in starch + glucose MRS medium. It is interesting to note that the residual starch concentration was 0.9 $g \cdot l^{-1}$ at 23 h in starch MRS medium.

Characterization of the amylolytic enzyme

Comparison of the HPLC profiles after starch hydrolysis by the cell-free extract and commercial amylolytic enzymes (*Aspergillus oryzae* α-amylase, Sigma A0273; potato β-amylase, Sigma A7005 and *A. niger* amyloglucosidase Sigma A3514; Sigma, Poole, Dorset, UK) was performed to characterize the amylolytic activity exhibited by *L. plantarum* A6. Under these conditions, the main products of starch hydrolysis analysed by HPLC were glucose from amyloglucosidase, maltose from β-amylase and a mixture of glucose, maltose and oligosaccharide (retention time of 5.2 min) from α-amylase. The breakdown profiles of starch by the enzyme from *L. plantarum* A6 was similar to that of α-amylase, thereby indicating that the enzyme synthetised by *L. plantarum* A6 is an extracellular α-amylase.

Effect of pH on enzymatic activity

Enzymatic activity was tested within the pH range 2.5–8.0 (Fig. 4). Maximum enzyme activity was observed at pH 5.0. Seventy percent of the activity was still observed at pH 4.0, but at pH 2.5 and 8.0 the enzyme was almost completely inactivated.

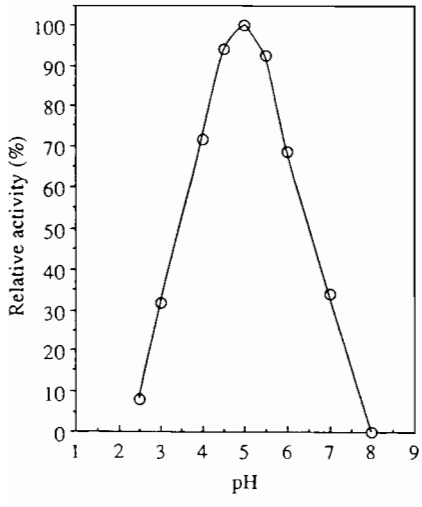

Fig. 4. Influence of pH on the amylase activity of *L. plantarum* A6 at 40° C

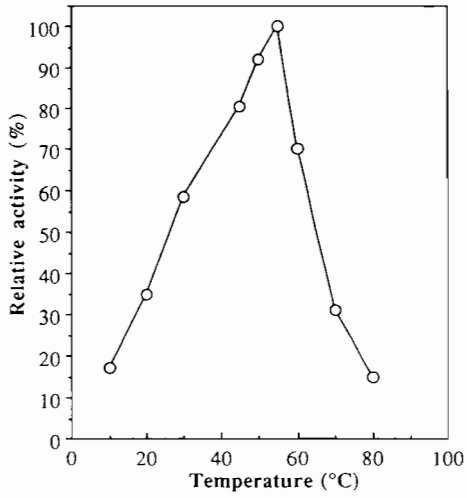

Fig. 5. Influence of temperature on the amylase activity of *L. plantarum* A6 at pH 6.0

Effect of temperature on enzymatic activity

The influence of temperature on the amylase activity tested within the range 10–80° C is presented in Fig. 5. The optimal temperature was 55° C under the conditions studied and 60% activity was still observed at 30° C.

Discussion

The presence of amylase in lactic acid bacteria has been already reported. However, to the best of our knowledge, no author has described any strains of amylolytic *L. plantarum*. Investigating the bacteria microflora of fermented cassava tubers, Regez et al. (1988) isolated numerous *L. plantarum* strains, but no amylolytic strains were reported. Recent studies (Scheirlinck et al. 1989) were carried out to integrate the α-amylase gene of *Bacillus stearothermophillus* in the genome of an *L. plantarum* strain. However, the expression, stability and competitiveness of the transformed strain in a natural medium remain to be verified.

In our work, a natural amylolytic strain of *L. plantarum* was isolated from cassava roots. The enzyme synthesized was identified as an α-amylase on the basis of HPLC analysis. However, a further purification stage would be necessary to ensure that this activity is not associated with other amylases (amyloglucosidase and β-amylase). Nevertheless, it is to be noted that only α-amylase activity has been described in the literature for amylolytic lactic acid bacteria.

Investigation of the properties of the enzyme indicated its extracellular and acidotolerant characteristics. Thus nearly 70% of the enzyme activity remains at pH 4.0, (a state attained after the 3rd day of retting (Okafor et al. 1984). These properties appear to be different from those observed in other amylolytic lactic acid bacteria. Indeed, the extracellular character was observed in *L. cellobiosus* (Sen and Chakrabarty 1986), but the

temperature and pH activity profiles of the enzyme synthesized by this microorganism are quite different to those of *L. plantarum* A6. In contrast, two *Lactobacillus* amylolytic lactic acid bacteria isolated from a chicken crop by Champ et al. (1983) have comparable profiles but the amylase synthesized by these two microorganisms were cell-linked.

The use of this strain can be envisaged in several fields of application. The high biomass yield and the high growth rate on starch mean that the microorganism could be used in starter cultures to improve the conservation and stability of starch-based fermented foods. The particularly high starch/lactic acid conversion yield (80%) also suggests the possibility of industrial lactic acid production from starch residues, though more investigation will be needed to evaluate its commercial viability. Since it has been shown that *L. plantarum* is capable of energy uncoupling (Giraud et al. 1991), this would simplify the procedure proposed by Kurosawa et al. (1988) in which a co-immobilized mixed culture system of *A. awamori* and *S. lactis* was proposed.

The data reported here would thus appear to show that this new lactic acid bacterium is of particular interest not only from a taxonomic point of view but also for its capacity to develop rapidly and massively in starch-based media. Further research will therefore be undertaken to purify the enzyme for better characterization of its biochemical properties, to monitor growth of this bacterium on raw starch and to determine the real improvement in quality and stability of foodstuffs resulting from mass inoculation of cassava tubers with the strain.

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ARTICLE 5

Giraud E, Gosselin L et Raimbault M.

Production of a *Lactobacillus plantarum* starter with linamarase and amylase activities for cassava fermentation.

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Production of a *Lactobacillus plantarum* Starter with Linamarase and Amylase Activities for Cassava Fermentation

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Abstract: *Lactobacillus plantarum* strain A6 isolated from cassava, cultured on cellobiose MRS medium showed a growth rate of 0·41 h^{-1}, a biomass yield of 0·22 g g^{-1}, and produced simultaneously an intracellular linamarase (76·4 U g^{-1} of biomass) and an extracellular amylase (36 U ml^{-1}). The synthesis of both enzymes was repressed by glucose. The use of such a strain as a cassava fermentation starter for gari production had the following influences: a change from a heterofermentative pattern observed in natural fermentation to a homofermentation, a lower final pH, a faster pH decline rate and a greater production of lactic acid (50 g kg^{-1} DM). However, this starter did not appear to play a significant role in cassava detoxification, since it was observed that the level of endogenous linamarase released during the grating of the roots was sufficient to permit the complete and rapid breakdown of linamarin.

Key words: fermented cassava, lactic acid bacteria, starter, *Lactobacillus plantarum*, amylase activity, linamarase activity, cyanide, *Manihot esculenta* Crantz, gari.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is an important staple food for 500 million people (Cock 1982). However, its use as a food is influenced by its toxicity. Cassava roots contain two cyanoglucosides, linamarin (2-(β-D-glucopyranosyloxyl) isobutyronitrile) and lotaustralin (2-(β-D-glucopyranosyloxyl) methylbutyronitrile)). Although much of these toxic components is removed during processing, in various foods a quantity still remains, depending on the process used (Nambisan and Sundaresan 1985). Daily consumption of these foodstuffs which still contain residual levels of cyanogenic compounds can result in chronic diseases such as goitre, cretinism, tropical atoxic neuropathy and tropical diabetes (Cock 1982).

During gari preparation, two stages appear to have a significant role in cassava detoxification (Nambisan and Sundaresan 1985; Vasconcelos *et al* 1990): (i) the grating or mincing of the roots permits, through the cell structure damage, the releasing of endogenous linamarase (EC 3.2.1.21, linamarin β-D glucoside glucohydrolase) able to hydrolyse linamarin into glucose and cyanohydrin (Conn 1969); and (ii) the roasting allows the removal of residual free cyanide (acetone cyanohydrin and HCN). On the other hand, the natural fermentation stage, through the development of lactic acid microflora, contributes to the sensory qualities of the final product (Ngaba and Lee 1979; Dougan *et al* 1983).

As reported by Mkpong *et al* (1989) and Ikediobi and Onyike (1982), the endogenous linamarase content could not permit the complete breakdown of linamarin. However, it was demonstrated (Ikediobi and Onyike 1982) that it is possible to reduce the gari toxicity by the addition of an exogenous linamarase during the fermentation. Many authors (Ikediobi and Onyike, 1982; Padmaja and Balagopal 1985; Okafor and Ejiofor 1990) have suggested the inoculation of fermenting cassava with a linamarase-producing microorganism. Bearing

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this in mind, the authors have recently reported that various lactic acid bacteria have the ability to hydrolyse linamarin (Giraud *et al* 1992). *Lactobacillus plantarum* strain A6, isolated from retted cassava for its amylolytic activity (Giraud *et al* 1991a), appeared to be the more suitable. Indeed, after culture on cellobiose MRS medium this strain showed a strong linamarase activity.

The purpose of this work was to study the physiology of this microorganism in order to produce a starter with high linamarase and amylase (EC 3.2.1.1, 1,4-α-D-glucan glucanohydrolase) activities and evaluate the potential for use of such a strain in cassava fermentation

EXPERIMENTAL

Strains

The microorganisms used were *Lactobacillus plantarum* Lacto-Labo (Dange Saint Romain, France) and *L plantarum* strain A6 isolated from retted cassava (Giraud *et al* 1991a). The strains were conserved in glycerol at −80°C in 1 ml Nunc tubes.

Media and culture conditions

The composition of the basic medium was identical to that of MRS medium (de Man *et al* 1960). For our purposes glucose was replaced by (a) cellobiose 20 g litre^{-1}; (b) cellobiose 10 g litre^{-1} and glucose 10 g litre^{-1}; and (c) cellobiose 10 g litre^{-1} and soluble starch 10 g litre^{-1} (Prolabo). Strains were cultured in 2-litre bioreactors (Biolaffite, Poissy, France) at 30°C and agitated to 200 rpm; pH was adjusted to 6·0 by addition of 5 M NaOH. Inoculation at 100 ml litre^{-1} was performed with a 20 h pre-culture in the same medium as used for fermentation.

Analytical methods

The biomass, the lactic acid, glucose and cellobiose contents, as well as total sugars were determined by the methods described by Giraud *et al* (1991b).

Enzyme assays

Amylase activity was assayed by adding 0·1 ml of culture supernatant to 0·8 ml of a solution containing 12 g litre^{-1} of Prolabo soluble starch (Paris, France) in 0·1 M citrate/phosphate buffer pH 5·5. The reaction was stopped by addition of 0·1 ml of 1 M H_2SO_4. After incubation at 55°C, residual starch at different time periods was determined colorimetrically at 620 nm by adding 0·1 ml of the reaction mixture to 2·4 ml of an iodine solution (KI, 1·2 g; I_2, 0·12 g; distilled water 1 litre). One enzyme unit is defined as the amount of enzyme that permits the hydrolysis of 10 mg of starch in 30 min under the conditions given above.

Linamarase activity was assayed on whole cells by the method described by Giraud *et al* (1992).

Assays on gari

Fresh imported cassava tubers from Cameroon were obtained from Anarex (Paris, France). Gari was prepared from peeled washed cassava roots, chopped and minced in a food mixer. The pulp obtained was packed tightly into plastic sterile screw-capped containers (60 ml; OSI, A12.160.56) and held at 30°C. Three batches were prepared: (a) natural fermentation, using the endogenous microflora present; (b) fermentation after inoculation with *L plantarum* A6 (10^8 cfu g^{-1} of dry cassava) previously cultured in bioreactors on cellobiose MRS medium; (c) fermentation after inoculation with *L plantarum* Lacto-Labo (10^8 cfu g^{-1} of dry cassava) previously cultured in bioreactors on MRS cellobiose. Cells were washed in physiological solution before cassava inoculation.

A container from each batch was monitored every day to test the parameters as follows.

- The pH was measured on a sample (10 g) homogenised in distilled water (20 ml). Moisture was measured by drying a sample (10 g) at 105°C for 24 h.
- The linamarase activity indigenous in the cassava was determined by the method of Giraud *et al* (1992) on the supernatant (0·1 ml) from a sample (10 g) homogenised in 10 ml 0·1 M phosphate buffer pH 6·0, chopped and mixed for 1 min at 4°C using an Ultra-Turrax (Janke and Kunkel, Ika-Werk, Staufen, Germany) and centrifuged (10000 × *g*, 10 min).
- Lactic acid bacteria (LAB) were estimated on a sample (10 g) homogenised in 90 ml of sterile physiological solution; colonies were counted on MRS agar using a spread plate technique on Petri dishes, after incubation (30°C, 48 h).
- To measure cyanide compounds and organic acids, 10 g of sample were homogenised in 10 ml 0·05 M H_2SO_4 and chopped and mixed for 1 min at 4°C using an Ultra-Turrax; the assays were carried out on the supernatant obtained from centrifugation.

Organic acids were determined by HPLC under the conditions described by Giraud *et al* (1991b). Cyanide compounds were measured by a procedure based on the method of Cooke (1978); HCN was assayed using a Merck Spectroquant Kit (ref. 14800) by omitting the linamarase (a pellet from 1 ml of an *L plantarum* A6 cultured on MRS cellobiose medium) and NaOH from

the assay Linamarin was determined as the difference between free and total cyanides, and cyanohydrin as the difference between free cyanides and HCN.

RESULTS AND DISCUSSION

Production of an *L plantarum* starter

Figures 1–3 represent growth kinetics, sugar consumption, and linamarase and amylase production of *L plantarum* A6 cultured on different media as indicated in the experimental section. The main fermentation parameters are shown in Table 1. In all three tested media, biomass productivity and growth rates were high and practically identical. However, linamarase and amylase amounts differed with the medium used.

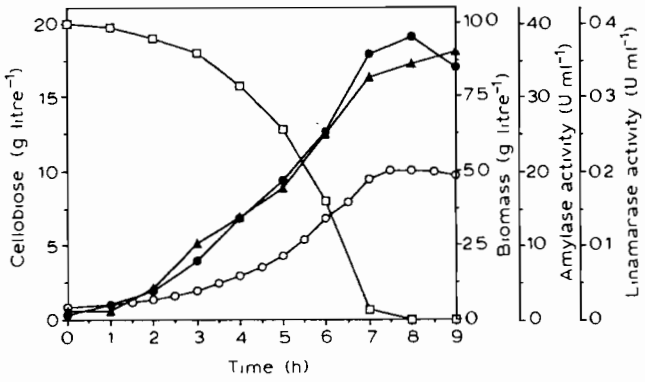

Fig 1. Fermentation of *L plantarum* A6 on cellobiose MRS medium at 30°C and pH 6·0: □, cellobiose; ○, biomass; ▲, amylase activity; ●, linamarase activity.

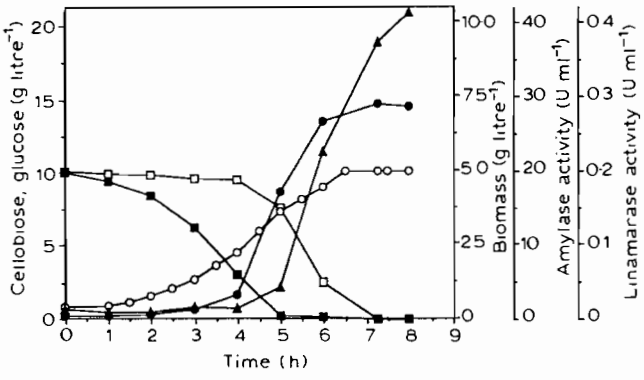

Fig 2. Fermentation of *L plantarum* A6 on cellobiose–glucose MRS medium at 30°C and pH 6·0: ■, glucose; □, cellobiose; ○, biomass; ▲, amylase activity; ●, linamarase activity.

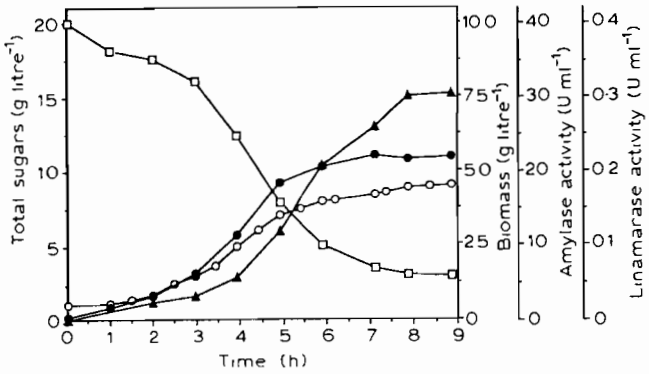

Fig 3. Fermentation of *L plantarum* A6 on cellobiose–starch MRS medium at 30°C and pH 6·0: □, total sugars, ○, biomass; ▲, amylase activity; ●, linamarase activity.

On cellobiose MRS medium

Linamarase and amylase synthesis occurred at the start of fermentation and seemed to be related to biomass formation. Linamarase concentration at the end of the fermentation was 76·4 U g^{-1} of biomass. A recent study (Giraud *et al* 1992), carried out in flasks on the same medium, demonstrated that the amount of linamarase produced was 29 U g^{-1} of biomass. In the bioreactor, under controlled conditions, it increased 2·6 times. It was noticed that the strain produced an amylase while there was no starch in the medium, moreover, the amount was higher than that obtained on cellobiose–starch MRS medium.

On cellobiose–glucose MRS medium

The kinetics indicated that glucose was rapidly consumed during the first 4 h of fermentation, while cellobiose content remained constant. It appeared that linamarase was not formed as long as the glucose concentration remained at about 3 g $litre^{-1}$. Extremely rapid uptake of

TABLE 1
Fermentation parameters of *L plantarum* A6 cultured on various media at pH 6·0 and 30°C

Medium	*Growth rate* (h^{-1})	*Biomass*		*Enzyme activity*	
		Concn. ($g\ litre^{-1}$)	*Yield* ($g\ g^{-1}$)	*Linamarase* ($U\ g^{-1}$)	*Amylase* ($U\ ml^{-1}$)
MRS cellobiose	0·41	5	0·22	76	36
MRS cellobiose–glucose	0·46	5	0·23	59	42
MRS cellobiose–starch	0·44	4·5	0·23	49	30

cellobiose was then observed and it correlated well with considerable synthesis of linamarase (β-glucosidase). Amylase synthesis was slightly retarded and occurred when the glucose concentration was approximately zero; about 80–90% of the enzymes was synthesised during this stage. It is interesting to note that the production of amylase reached a level higher than that observed on the two other tested media.

Glucose had a repressive effect on the synthesis of the two enzymes and the effect was more significant on amylase synthesis. Previous work by Abalaka and Garba (1989) also provided evidence of the strong influence of glucose content on the linamarase production for various fungi. This repressive effect of glucose on the amylase synthesis has already been reported (Giraud *et al.* 1991a).

On cellobiose–starch MRS medium

The growth profile differed from those in the other two media tested, and the biomass production was slightly lower (10%). A linear growth phase was observed after 5 h of fermentation and it seemed to be related to a difficulty on the part of the microorganism to utilise starch degradation by-products with consequent retardation of total sugar consumption.

Concerning enzyme synthesis, linamarase and amylase production was noted from the initial stages of the fermentation. Nevertheless, while linamarase production appeared to be related to the biomass formation as observed on cellobiose MRS medium, amylase was produced mainly during linear growth. Thus, the amount of amylase produced rose by 250% while biomass only increased by 30%.

This study showed that the substrate used influenced strongly the amounts produced, and the production kinetics, of both linamarase and amylase. The high biomass yield, and the strong linamarase and amylase activities observed after culture on cellobiose MRS medium, suggests that *L plantarum* strain A6 can be used as a very suitable starter for cassava fermentation.

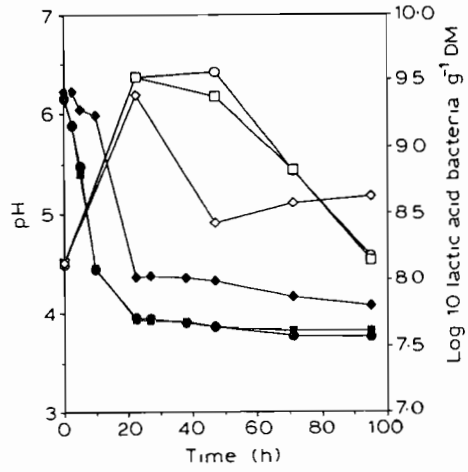

Fig 4. Changes in pH and numbers of lactic acid bacteria (LAB) during cassava fermentation. Natural fermentation (◆, pH; ◇, LAB); inoculated with *L plantarum* A6 (●, pH; ○, LAB); inoculated with *L plantarum* Lacto-Labo (■, pH; □, LAB).

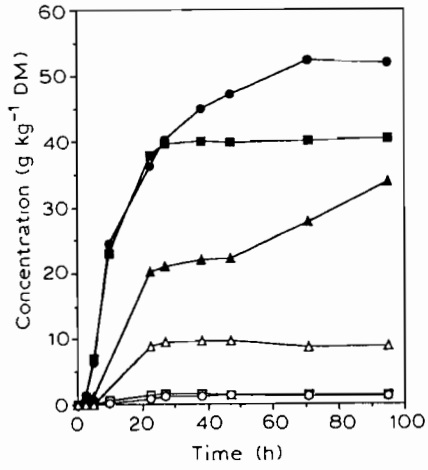

Fig 5. Evolution of lactate and acetate concentration during cassava fermentation. Natural fermentation (▲, lactate; △, acetate); inoculated with *L plantarum* A6 (●, lactate; ○, acetate); inoculated with *L plantarum* Lacto-Labo (■, lactate; □, acetate).

Inoculation effect of *L plantarum* on cassava fermentation

Three different types of fermentation were carried out: (a) natural cassava fermentation, (b) cassava inoculated with *L plantarum* A6, (c) cassava inoculated with a control strain *L plantarum* Lacto-Labo.

Variation of pH, organic acids and LAB

In all three types, a rapid pH decrease was observed from the start (Fig 4). The naturally fermented cassava (type 1) showed a steep fall from 6·2 to 4·3 and types 2 and 3 both fell from 6·2 to 3·9. This pH shift was correlated with lactic acid, the principal metabolite produced (Fig 5). These data confirm that the LAB are the predominant fermentative microflora, reaching 5×10^9 cfu g^{-1} after 24 h of fermentation in all three types of fermentation (Fig 4).

In natural cassava fermentation simultaneous production of lactic and acetic acids, and traces of propionic and butyric acids and ethanol, were observed within the first 24 h. However, while the acetate content reached its maximum level (10 g kg^{-1} DM) and remained constant after the first day of fermentation, an increase in lactate concentration was noticed from the second day. This may suggest that natural fermentation was initiated by heterolactic bacteria and was later supplanted by homolactic bacteria which are more acid-tolerant. This hypothesis is in agreement with the results of Oyewole

and Odunfa (1990) who reported a predominant development of *Leuconostoc mesenteroides*—in the study of characterisation and distribution of the lactic acid microflora during the preparation of fufu—replaced subsequently by *L plantarum*. Therefore, they suggested that this sequence is due to the inability of *L mesenteroides* to tolerate an increase in acidity.

In the inoculated fermentations the lactic acid content was higher. The production kinetics of this acid were identical for both *L plantarum* tested strains during the first 24 h. However, while for the control strain this concentration reached its maximum (40 g kg^{-1} DM) and then remained at that level, for the amylolytic strain (*L plantarum* A6) we found that the lactate production continued to rise, increasing by 25%.

Likewise, in inoculated fermentation assays, traces of ethanol, propionate and butyrate were found. Furthermore, the lower acetate production showed that a massive inoculation with an *L plantarum* strain inhibited the natural development of heterolactic microflora.

Development of cyanide compounds and endogenous linamarase

In all three types of fermentation cyanides, present initially as linamarin, were transformed in less than 5 h to acetone cyanohydrin and HCN (Fig 6). The amount of free cyanide then remained constant until the end of the fermentation. Nevertheless, in the inoculated fermentations, it was observed that the proportion of acetone cyanohydrin was higher. This phenomenon appears to be related to a faster pH decrease that results, as reported by Cooke (1978), in a lower rate of acetone cyanohydrin breakdown to acetone and HCN.

Here, it appeared that the amount of cassava-indigenous-linamarase released during the grating stage was sufficient to permit the complete and rapid hydrolysis of linamarin. It was observed that this enzymatic activity decreased during the fermentation period (Fig 7). In inoculated fermentations this was more significant and may be associated with protein hydrolysis by *L plantarum* or by a higher and faster pH decrease promoting denaturation of cassava-indigenous-linamarase.

The work reported here showed that the inoculation of cassava pulp with a strain of LAB possessing a strong linamarase activity does not appear to contribute to cassava detoxification, and may have a mostly antagonistic effect. This result is in agreement with Vasconcelos *et al* (1990) who reported that 95% of initial linamarin is hydrolysed 3 h after grating the roots. In contrast, it differs from those showed by Ikediobi and Onyike (1982) and Okafor and Ejiofor (1990) who observed that linamarase addition, or the inoculation with a strain having linamarase activity, may improve detoxification. The observed differences may be explained by the use of non-traditional means for the preparation of gari, particularly during the grating of the

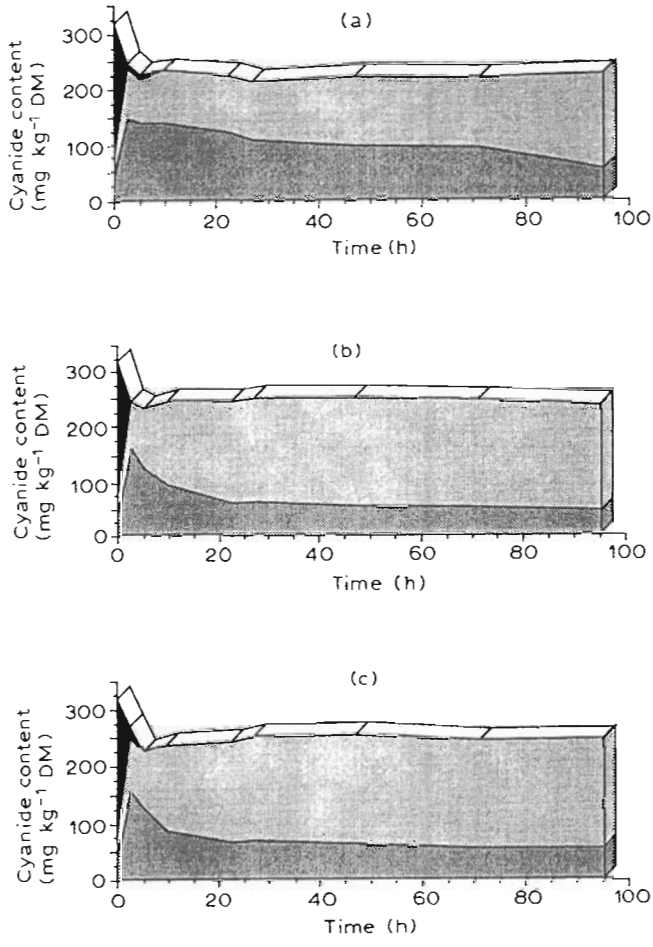

Fig 6. Changes in linamarin (dark shading), cyanohydrin (light shading) and HCN (medium shading) during cassava fermentation. (a) Natural fermentation; (b) inoculated with *L plantarum* A6; (c) inoculated with *L plantarum* Lacto-Labo.

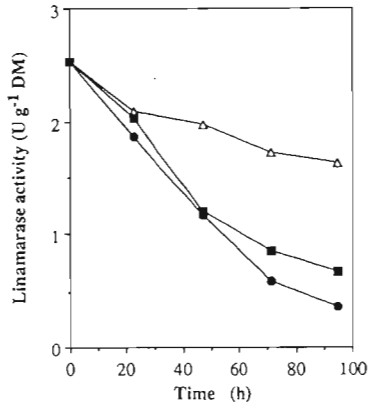

Fig 7. Change in linamarase activity during cassava fermentation. △, Natural fermentation; ●, inoculated with *L plantarum* A6; ■, inoculated with *L plantarum* Lacto-Labo.

cassava roots or as Mkpong *et al* (1990) noticed, by the utilisation of cassava varieties showing different levels of indigenous linamarase.

However, despite this, our work demonstrates that the utilisation of *L plantarum* A6 as a starter may play a

significant role in the development of sensory qualities, and in the standardisation and preservation of the final product by the large amounts of lactic acid produced and the resultant faster and more significant pH decrease.

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ARTICLE 6

Giraud E, Gosselin L, Marin B, Parada J.L, et Raimbault M.

Purification and characterization of an extracellular amylase activity from *Lactobacillus plantarum* strain A6.

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Purification and characterization of an extracellular amylase from *Lactobacillus plantarum* strain A6

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E GIRAUD, L GOSSELIN, B MARIN, J L. PARADA AND M RAIMBAULT 1993. Extracellular amylase from *Lactobacillus plantarum* A6 was purified by fractionated precipitation with ammonium sulphate and by anion exchange chromatography. The homogeneity of the purified fraction was tested by polyacrylamide gel electrophoresis and showed multiple amylase forms. A major form had an estimated molecular weight of 50 kDa. It was identified as an α-amylase, with an optimum pH of 5·5, an optimum temperature of 65°C and K_m value of 2·38 g l^{-1} with soluble starch substrate. The enzyme was inhibited by *N*-bromosuccinimide, iodine and acetic acid. The enzyme activation energy was 30·9 kJ mol^{-1}.

INTRODUCTION

Lactic acid bacteria can rarely convert starch into lactic acid. The main such strains identified are *Streptococcus bovis*, *Strep. equinus*, *Lactobacillus amylophilus*, *Lact. amylovorus*, *Lact. acidophilus* and *Lact. cellobiosus* (Nakamura and Crowell 1979; Nakamura 1981; Sen and Chakrabarty 1984; Kandler and Weiss 1986; Hardie 1986). Others, isolated from animal digestive tracts, have been described as amylolytic lactic acid bacteria (Champ *et al.* 1983; Cotta 1988). Practically no information is available about the characteristics of these enzymes. To the best of our knowledge, only the research by Lindgren and Refai (1984) and Sen and Chakrabarty (1986) covered the characterization of an amylolytic activity produced by a lactic acid bacterium.

A wild strain of *Lact. plantarum* (strain A6) was isolated from retted cassava and examined for its ability to break down starch (Giraud *et al.* 1991). The strain possesses particularly interesting characteristics. It is the first time that a *Lact. plantarum* amylolytic strain has been described and it was found that it synthesizes large amounts of extracellular α-amylase and its growth kinetics on glucose or starch-based media are similar.

More detailed investigation of this enzymatic activity was carried out because of the original features of the micro-organism and there has been only a limited amount of research on the amylolytic activities of lactic acid bacteria. The purification and characterization of the extracellular amylolytic activity excreted by *Lact. plantarum* A6 are described here.

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MATERIALS AND METHODS

Organism

Lactobacillus plantarum strain A6 isolated from retted cassava was used (Giraud *et al.* 1991). The strain was preserved in glycerol at −80°C.

Medium and culture conditions

The medium composition was identical to that of MRS medium (De Man *et al.* 1960) except that glucose was replaced by 2% soluble starch (Prolabo, Paris, France) and $CaCl_2$ was added at 0·5 g l^{-1}. The bacterium was cultured in a 2 l bioreactor (LSL-Biolafitte, Saint Germain en Laye, France) at 30°C and agitated at 200 rev min^{-1}. The pH was adjusted to 6·0 by the addition of 5 mol l^{-1} NaOH. The reactor was inoculated with 10% (v/v) of 20-h preculture in the same medium used for fermentation.

Amylase assay

α-Amylase activity was measured by incubating 0·1 ml of appropriately diluted enzyme solution with 0·8 ml of a solution containing 1·2% of Prolabo soluble starch in 0·1 mol l^{-1} citrate-phosphate buffer, pH 5·5 at 55°C. The reaction was stopped by the addition of 0·1 ml of 1 mol l^{-1} H_2SO_4. After incubation, residual starch contents after different lengths of time were determined colorimetrically at 620 nm by adding 0·1 ml of the reaction mixture to 2·4 ml of an iodine solution containing (g l^{-1} in distilled water): KI, 30; I_2, 3, diluted to 4%. One enzyme unit is defined as the amount of enzyme that permits the hydrolysis of 10 mg of starch in 30 min under the conditions described above. The protein concentration was estimated using the method described by Bradford (1976) with a Biorad Kit (Cat no. 500-0001, Ivry Sur Seine, France) with bovine serum albumin as standard.

Purification of amylase

Fermentation was stopped after culture for 9 h. After the removal of cells by centrifugation (15 000 *g* for 15 min, 4°C), the supernatant fluid (750 ml) was filtered through a cellulose filter (0·45 μm pore size, HAWP type; Millipore, Saint Quentin les Yvelines, France) to remove cell debris. Powdered ammonium sulphate was then added slowly to the supernatant fluid with constant stirring at 4°C. Most of the amylase activity was precipitated at between 50 and 70% saturation. After ammonium sulphate fractionation, the precipitated protein collected by centrifugation (15 000 *g* for 30 min, 4°C) was resuspended in 50 mmol l^{-1} KH_2PO_4/Na_2HPO_4 buffer pH 6·8 (standard buffer). The enzyme solution was washed, concentrated with a PM-10 Amicon ultrafiltration membrane and loaded onto a DEAE-cellulose column (DE-52; Whatman Laboratory Sales, Hillsboro, Oregon, USA) (25 × 250 mm, flow rate 2·5 ml min^{-1}, 25°C) previously equilibrated with the standard buffer. The enzyme was eluted with a concave sodium chloride gradient (0–1·0 mol l^{-1}) and fractions (5 ml) were collected. The fractions that were enzymatically the most active were pooled, dialysed overnight at 4°C against the standard buffer and used for further studies. They were kept at −30°C. No activity was lost for at least 3 months under such conditions.

Polyacrylamide gel electrophoresis

This was carried out according to the method of Laemmli (1970) with a 10% running gel and 4% stacking gel. Electrophoresis under non-denaturing conditions was performed in the absence of sodium dodecyl sulphate and β-mercaptoethanol in any buffer. Gels were run at a constant 150 V for 1 h at 25°C. Proteins were stained by the silver method of Oakley *et al.* (1980).

Amylase stain

After electrophoresis, the gel was incubated for 1 h at 30°C in 0·1 mol l^{-1} citrate-phosphate buffer, pH 5·5, containing 1% soluble starch. After two washes with distilled water, light lanes (representing starch hydrolysis areas of amylase activity) were detected by immersing the gel in Lugol's solution.

Molecular mass determination

SDS-PAGE electrophoresis was used to determine the approximate molecular mass of amylase. Myosin (200 000), β-galactosidase (116 250), phosphorylase-b (97 400), bovine serum albumin (67 000) and ovalbumin (45 000) served as marker proteins (Biorad, Cat no. 161-0315).

Kinetics

The Michaelis constant (K_m) of the purified enzyme was determined with different concentrations of soluble starch as substrate. Values were obtained from double reciprocal plotting as described by Lineweaver and Burk (1934).

Optimum pH and temperature

The effect of pH on the enzyme activity was studied in a 3–7·5 pH range with 0·1 mol l^{-1} citrate-phosphate buffer at 55°C. The enzymatic activity profile according to temperature was determined within a 10°–80°C temperature range under standard conditions (see above).

RESULTS

Purification of amylase

The results of the purification of amylase produced by the strain *Lact. plantarum* A6 are summarized in Table 1. The first step in purification was conventional $(NH_4)_2SO_4$ frac-

Table 1 Purification of α-amylase of *Lactobacillus plantarum* (strain A6) cultivated in a modified MRS medium containing 2% (w/v) soluble starch and 0·5 g l^{-1} $CaCl_2$ at 30°C

Materials	Volume (ml)	Protein (mg)	Activity (U)	Specific activity U mg^{-1}	Yield (%)	Purification (fold)
Culture filtrate	750	82·5	35 100	425	100	1
$(NH_4)_2SO_4$ (50–70% fraction)	39	18·1	25 935	1433	73·9	3·4
Ultrafiltrate	8·8	10·4	16 016	1540	45·6	3·6
DEAE cellulose (117–130 fractions)	61·8	1·5	12 484	8270	35·6	19·5

tionation. The 50–70% fraction revealed maximum enzyme activity and was selected for further purification by DEAE-cellulose. The elution profile (Fig. 1) displayed only one amylase activity peak. The enzyme was eluted at 0·44–0·52 mol l^{-1} NaCl. The maximum purification level achieved was about 20-fold and the recovery yield was approximately 35%. Enzyme purity and efficiency of the proposed purification procedure were estimated by polyacrylamide gel electrophoresis under non-denaturing conditions. The samples from the different purification steps were loaded onto the gel in such a way as to deposit constant amylasic activity (7 U per lane). The sensitivity of the technique used to visualize protein (silver staining) made it possible to assess the effectiveness of the method used for purifying the enzymatic activity (Fig. 2).

A major band was shown in the purified fraction (lane 3) together with three low intensity sub-bands spaced at regular intervals. Lugol visualization (lane 4) after incubation of the gel with starch showed that the amylasic activity was sited principally at the main band level but was also present at a weaker level in the sub-bands. It was noted that these three bands of minor activity were initially observed in the crude extract and were thus not formed during the purification procedure.

Molecular mass

In SDS-PAGE analysis (Fig. 3), a well-defined band for a molecular weight of 50 kDa and a diffuse band with a molecular weight of close to 150 kDa were observed. The same electrophoretic profiles were found in the presence or absence of β-mercaptoethanol (results not shown).

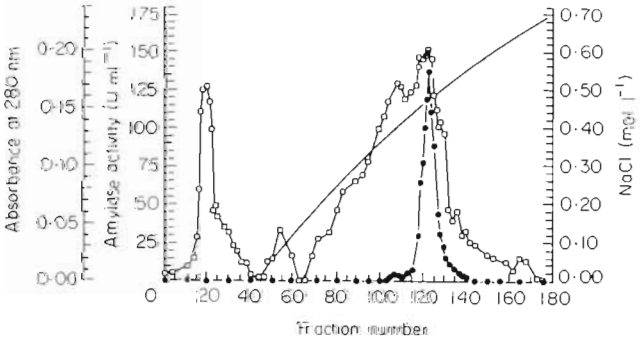

Fig. 1 Elution profile of *Lactobacillus plantarum* α-amylase on DEAE-cellulose column chromatography. The ultrafiltered enzyme solution obtained after ammonium sulphate precipitation was applied to the column. The column was equilibrated with phosphate buffer (pH 6·8) and the sample eluted with an NaCl gradient (0–1·0 mol l^{-1}) in phosphate buffer. Fractions (5 ml) were collected. □, Absorbance at 280 nm; ●, amylase activity; —, NaCl concentration

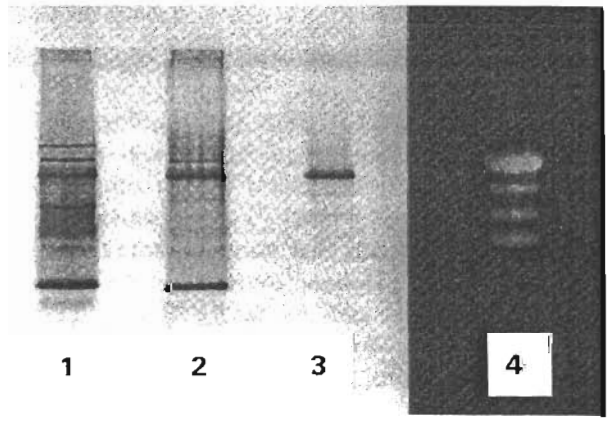

Fig. 2 Polyacrylamide gel electrophoresis of active fractions obtained from each purification step. Lane 1, Cell-free extract; lane 2, after ammonium sulphate precipitation; lane 3, after DEAE-cellulose chromatography; lane 4, after DEAE-cellulose chromatography, the bands supposed to be α-amylase were assumed by their starch-hydrolysing activity

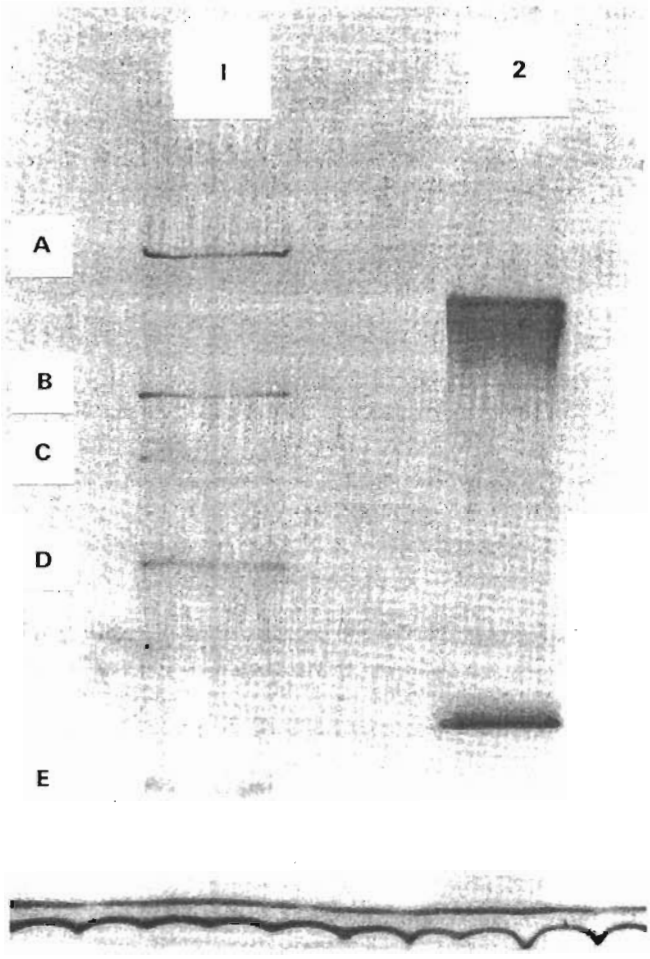

Fig. 3 SDS-polyacrylamide gel electrophoresis of purified α-amylase. Lane 1, Standard proteins (Biorad): A, myosin (MW 200 000); B, β-galactosidase (MW 116 250); C, phosphorylase b (MW 92 500); D, bovine serum albumin (MW 66 200); E, ovalbumin (MW 45 000). Lane 2, Purified α-amylase

Starch hydrolysis

The mode of action of the purified enzyme on soluble starch was examined by simultaneously measuring the decrease in iodine-staining power and the production of reducing sugars from starch using the method of Miller (1959). Starch hydrolysis by the enzyme (Fig. 4) was accompanied by a rapid reduction in the iodine-staining capacity of the substrate with correspondingly slow release of reducing sugar. Such a pattern is typical of an endo-attacking enzyme cleaving 1,4-glucosidic linkages.

Effects of pH on enzyme activity

The enzyme acts preferentially at an acid pH with an optimum at pH 5·5 (Fig. 5). Activity loss at pH levels of

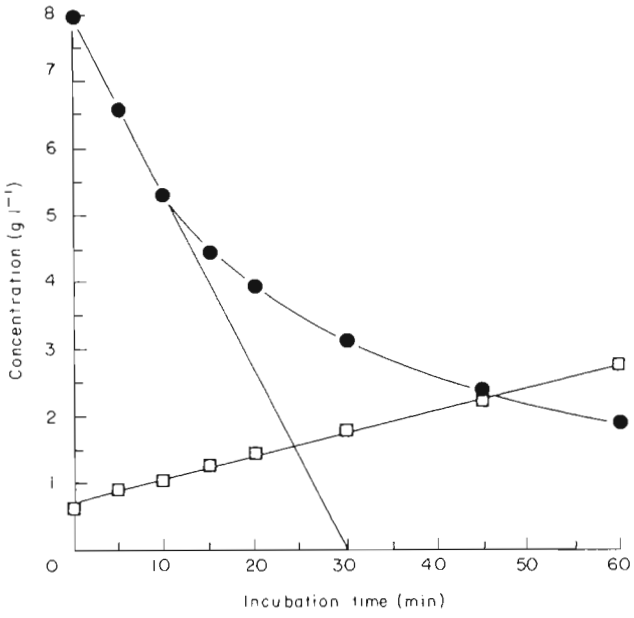

Fig. 4 Reduction in iodine-staining and the release of reducing power from starch by the purified enzyme at pH 5·5 and 55°C. ●, Concentration of starch determined by iodine staining; □, concentration of reducing power

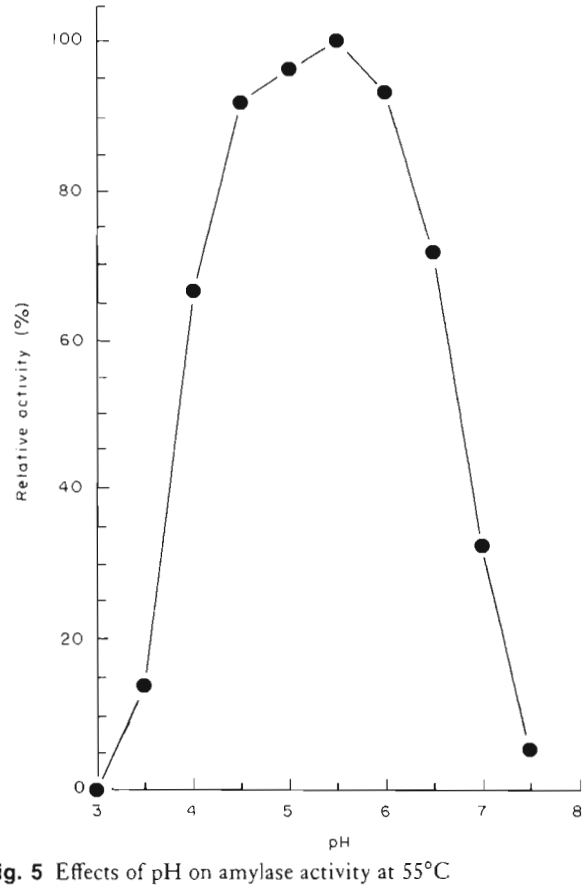

Fig. 5 Effects of pH on amylase activity at 55°C

4·5–6 did not exceed 10–15%. Activity falls rapidly outside this range, decreasing to zero close to pH 3 and 8. Activity is almost totally retained for 5 d at ambient temperature. However, at extreme pH levels, and especially at pH 3·8, the enzyme was unstable and 80% loss of activity was observed over a 5 d period.

Effects of temperature on enzyme activity

The influence of temperature on the amylase activity in 0·1 mol l^{-1} citrate-phosphate buffer, pH 5·5, tested within the 10°–80°C range is shown in Fig 6. The optimal temperature was 65°C The activation energy of the enzyme was 30·9 kJ mol^{-1} as calculated from the Arrhenius plot

Michaelis constant (K_m)

The enzyme K_m value was determined with soluble starch as substrate and was found to be 2·38 g l^{-1} at 30°C and pH 5·5 from the Lineweaver–Burk plot.

Action of cations

The effect of cations at 0·01 mol l^{-1} were all tested as chloride or sulphate, as these two ions have no effect on enzymatic activity. The results are displayed in Table 2 and show that the enzyme was strongly inhibited by mercury (100% at the concentration used). No other significant activation or inhibition was observed with the other cations.

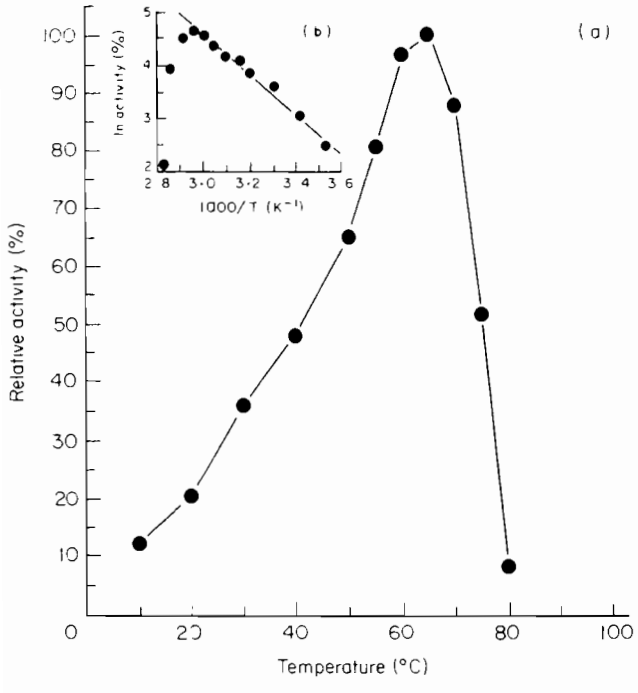

Fig. 6 Effects of temperature on amylase activity at pH 5·5. (a) Relative activity *vs* temperature; (b) Arrhenius plot

Table 2 Influence of cations (0·01 mol l^{-1}) on amylase activity

Cation	% activity
$BaCl_2$, $2H_2O$	103
$CuSO_4$	109
$MgSO_4$, $7H_2O$	104
$CoCl_2$, $6H_2O$	100
$NiCl_2$, $6H_2O$	109
$HgCl_2$	0
$ZnSO_4$, $7H_2O$	107
$FeSO_4$, $7H_2O$	93
$CaCl_2$, $2H_2O$	95
KCl	114
$FeCl_3$	115
CdN_2O_6, $4H_2O$	98
$SnCl_2$, $2H_2O$	89

Action of other effectors

The other effectors tested at concentrations of 10^{-2} or 10^{-3} mol l^{-1} are shown in Table 3. Strong inhibition by *N*-bromosuccinimide and partial inhibition with 4-dimethylaminobenzaldehyde were found, indicating the

Table 3 Influence of effectors on amylase activity

Effector	Concentration (mol l^{-1})	% activity
Amino-5-tetrazole	10^{-2}	112
4-Dimethylaminobenzaldehyde	10^{-2}	70
Iminodiacetic acid	10^{-2}	118
Woodward reagent	10^{-2}	102
EDTA	10^{-2}	91
N-acetylacetone	10^{-2}	100
N-ethylmaleimide	10^{-2}	90
2, 5-Dimethoxytetrahydrofuran	10^{-2}	111
Trifluoroacetic anhydride	10^{-2}	89
Acetic acid	10^{-2}	82
Iodine	10^{-3}	6
Iodine/tyrosine	$10^{-3}/5 \times 10^{-3}$	79
N-bromosuccinimide	10^{-3}	0
N-bromosuccinimide/tryptophan	$10^{-3}/5 \times 10^{-3}$	63

presence of a tryptophan group at the active site. Inhibition by iodine suggests the action of a phenol group and probably tyrosine The simultaneous addition of amino acid (tryptophan with *N*-bromosuccinimide in the first case and tyrosine with iodine in the second case) limited the extent of the observed inhibition. The decrease in activity measured after the addition of acetic acid suggests the action of an amino acid with an OH group (serine and/or threonine).

DISCUSSION

The purification procedure described above makes it possible to obtain, in only two stages, a protein fraction containing most of the amylasic activity of *Lact. plantarum* A6 enriched by a factor of nearly 20. Testing the homogeneity of the fraction by electrophoresis under native conditions revealed a major protein and three others that were quantitatively unimportant. However, all the proteins detected in the purified fraction possessed an amylasic activity. It was considered that these procedures were sufficient for purifying the extracellular amylasic activity of *Lact. plantarum* A6. SDS-PAGE analysis of the purified fraction resulted in distribution between a clearly defined band (50 kDA) and a diffuse band with a molecular weight of close to 150 kDa.

Several hypotheses can be put forward to account for these many amylase forms. The one we find most satisfactory is the suggestion that the purified extract consists of a population of aggregates of a 50 kDa amylase. This interpretation is based on the fact that most of the bacterial amylases described have a molecular weight of this order (Fogarty 1983). This type of aggregation of purified enzyme was observed in *Bacillus subtilis* amylase (Robyt and Ackerman 1973), with zinc as the factor inducing clumping. The factor remains to be defined in our case.

Further experimental studies would be required to support this hypothesis. The amount of enzyme isolated was not large enough for further investigation. Immunological characterization would probabably determine the type of relation between the different amylase forms observed and thus confirm the hypothesis.

With regard to the characteristics of the lactic acid bacterial amylases described in the literature, the properties of the enzyme synthesized by *Lact. plantarum* A6 are different. The enzyme from a *Leuconostoc* spp. studied by Lindgren and Refai (1984) had a pH optimum of 6·0 and a temperature optimum of 40°C. Two active enzyme fractions were clearly separable by isoelectric focusing. The enzyme isolated from *Lact. cellobiosus* (Sen and Chakrabarty 1986) had a molecular weight of 22·5–24 kDa, a pH optimum from 6·3 to 7·9 and a temperature optimum of 40–50°C. However, compared with the characteristics of the other bacterial amylases, the properties of amylase of *Lact plantarum* A6 are very similar to those of *B. subtilis* (Fischer and Stein 1960; Welker and Campbell 1967; Robyt and Ackerman 1973; Fogarty 1983), i.e. extracellular enzyme, identical optimum pH, identical optimum temperature, presence of tyrosyl phenolic groups at the active site and presence of multiple forms (aggregates).

The exceptional capacity of *Lact. plantarum* A6 to break down starch led us to consider the acquisition of this feature Might it result from the transfer of genetic material between *B subtilis* and *Lact. plantarum*, as both are microorganisms in the natural microflora of fermented cassava (Nwanko *et al.* 1989) and their amylasic activities we found to be very similar? Closer investigation would enable us to answer this question.

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ARTICLE 7

Giraud E, Champailler A et Raimbault M.

Degradation of raw starch by a wild amylolytic strain of *Lactobacillus plantarum*

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Degradation of Raw Starch by a Wild Amylolytic Strain of *Lactobacillus plantarum*

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***Lactobacillus plantarum* A6, isolated from fermented cassava, can break down cassava raw starch that has not been subjected to preliminary physicochemical treatment. When the pH was kept at 6, the microorganism cultured in a bioreactor excreted a high α-amylase activity (60 U/ml). Synthesis of the enzyme occurred during the stationary phase and resulted in full hydrolysis of the cassava starch granules. This gave 41 g of lactic acid from 45 g of raw starch after 3 days of fermentation. Enzymatic attack was evident under scanning electron microscopy in the rougher appearance of the surface of starch granules and in the presence of large cavities in some of them. In contrast, when the pH was not regulated, only a small amount of α-amylase activity was produced (2 U/ml) and no decrease in the starch content of the medium was observed. However, under scanning electron microscopy, some granules displayed a rougher surface, which might have been the result of weak enzymatic attack.**

Lactobacillus plantarum is a lactic acid bacterium common in numerous natural fermentation processes, such as those of silage, cabbage, cucumber, olive, cassava, etc. According to McDonald et al. (15), its ability to maintain a pH gradient between the inside and the outside of the cell in the presence of a large amount of acetate or lactate may explain why the bacterium can withstand acidified media and completes these fermentation processes. It converts low-molecular-weight sugars almost quantitatively into lactic acid, thus contributing to organoleptic qualities and the preservation potential of fermentable products. The bacterium is recommended as a starter culture in many cases for fermentation control and to obtain products of even quality (4, 16, 21, 23, 25, 27).

In the case of silage, the quantity of fermentable sugar is sometimes too small to ensure rapid production of a stabilizing amount of lactic acid. This can be overcome by the addition of other sources of fermentable carbohydrates, such as molasses, whey, or starch combined with α-amylase (26). Scheirlink et al. (22) proposed the use of the strain *L. plantarum* transformed by electroporation with plasmids containing the α-amylase gene of *Bacillus stearothermophilus* and of the endoglucanase of *Clostridium thermocellum*. However, use of such a genetically engineered strain under natural fermentation conditions may raise legal or ecological problems.

A wild strain of *L. plantarum* (strain A6) was recently isolated from retted cassava and selected for its ability to break down soluble starch (8). This was the first description of an *L. plantarum* amylolytic strain, and it was found that it synthesizes large amounts of extracellular α-amylase. A more detailed investigation of this enzymatic activity was carried out because of the original features of the microorganism (9). However, all of these studies were performed with soluble starch, whereas in nature, starch is found in a crystalline insoluble form which makes it less available to enzymatic hydrolysis. Work was therefore carried out to determine the capacity of the strain *L. plantarum* A6 to break down raw starch. This information would be useful in evaluating the potential for the utilization of *L. plantarum* A6 as a starter in certain traditional fermentation processes.

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MATERIALS AND METHODS

Organism. The microorganism used was *L. plantarum* A6 isolated from retted cassava (8). The strain was conserved in glycerol at −80°C.

Medium and culture conditions. The basal liquid nutrient medium contained 10 g of soy peptone obtained by papain digestion, 0.5 g of K_2HPO_4, 0.5 g of KH_2PO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of $MnSO_4 \cdot H_2O$, 0.5 g of $CaCl_2 \cdot 2H_2O$, and 1,000 ml of distilled water. The medium was sterilized at 121°C for 20 min, cooled to about 30°C, and mixed with the starch. The latter was obtained from roots of cassava plants (*Manihot esculenta* cv. Ngansa) harvested in the Brazzaville region 15 months after planting. Two types of starch were prepared. (i) Dry-heated starch was prepared as follows. The roots were peeled, diced, frozen at −80°C, and freeze-dried for 48 h. The flour obtained by grinding and sieving, with a 5% moisture content, was autoclaved (121°C, 20 min). (ii) Raw starch was prepared as follows. The roots were peeled and ground with a food mixer. The resulting pulp was sieved to remove fibers and homogenized with the basal liquid nutrient medium. The media thus prepared were transferred to a previously autoclaved bioreactor. The strain was cultured in a 2-liter bioreactor (Biolafitte, Poissy, France) at 30°C and agitated at 300 rpm. The pH was adjusted to 6.0 by the addition of 5 N NaOH. Inoculation of 0.01% (vol/vol) dry-heated starch and 10% raw starch was performed with a 20-h preculture. The medium used for the inoculum was identical to that of MRS medium (5) except that glucose was replaced by 2% soluble starch (Prolabo, Paris, France).

Estimation of the bacterial population. The bacterial population was monitored by use of a miniaturized most-probable-number method derived from that developed by Hernandez et al. (12) and adapted for counting lactic acid bacteria. The culture medium was API 50CHL medium (reference 50400; Biomérieux, Craponne, France) complemented with 10 g of glucose per liter. It was placed in 9-ml tubes and autoclaved

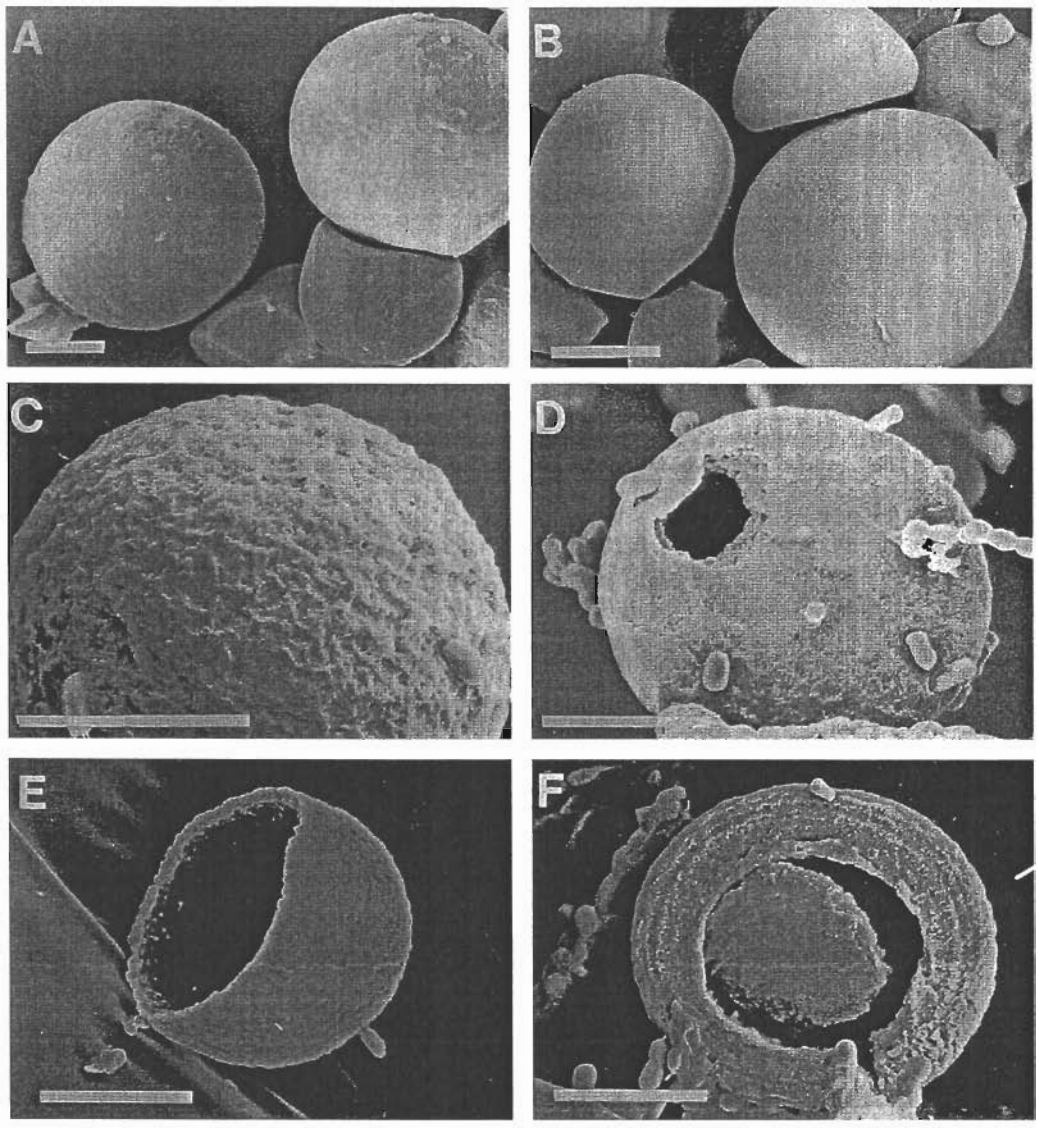

FIG. 1. SEMs showing the effect of *L. plantarum* A6 on cassava starch granules. (A) Dry-heated starch; (B) raw starch; (C, D, E, and F) raw starch granules after fermentation for 2 days with controlled pH. Bars, 5 μm.

(121°C, 20 min). Decimal dilutions of the sample to be counted were performed directly in the medium and placed on sterile microtitration plates (96 wells) at 200 μl per well and 12 wells per dilution. After incubation for 48 h at 30°C, the wells whose color had changed from purple to yellow were counted as positive. A computer program developed by Institut Pasteur, Lille, France (12), was then used to evaluate the most probable number of bacteria according to the number of positive wells.

Analytical methods. Raw starch contents were estimated by measurement of the dry weight obtained after two washing and centrifugation cycles and drying at 105°C for 24 h. Lactic acid was determined in the supernatant by high-performance liquid chromatography (HPLC) using an Aminex HPX 87H column (Bio-Rad Laboratories, Richmond, Calif.) with 0.006 M H_2SO_4 at a flow rate of 0.8 ml/min, at 65°C, and with refractive index detection.

Assay of α-amylase activity. α-Amylase activity was assayed by observing degradation of starch by measurement of its iodine-complexing ability. One enzyme unit is defined as the amount of enzyme that permits the hydrolysis of 10 mg of starch in 30 min under the conditions described by Giraud et al. (9).

Scanning electron microscopy. Sample preparation consisted of four steps: fixation by glutaraldehyde to prevent deformation of the structure, complete dehydration of the material, total removal of ethanol (dehydrating agent) by application of CO_2 at the critical point, and homogeneous gold metallization. The methodology used was similar to that reported by Blaha and Paris (1). SEM examination was performed with a JEOL JSM-6300F microscope (Université de Montpellier II).

RESULTS

Fermentation of *L. plantarum* A6 on dry-heated cassava starch. SEM observation showed that after treatment (freezing to −80°C, freeze-drying, and dry autoclaving), the starch granules had a crystalline appearance very similar to that of raw starch

granules. Although the granule surface was rougher than that of the raw starch granules (Fig. 1A and B), the preparation treatment did not appear to have caused considerable modification to the granule structure. However, the treatment reduced the endogenous microbial population from 10^5 to 10^1 bacteria per g of cassava flour. This reduced contamination risk and growth of *L. plantarum* A6 could be monitored by inoculating starch which had conserved its crystalline structure with a very low concentration of the bacterium (0.01%).

Figure 2 shows growth kinetics, starch degradation, and lactic acid and amylase production by *L. plantarum* A6 cultured in a fermentor on dry-heated starch with the pH set at 6. The initial starch (88 g/liter) was converted entirely into lactic acid (0.91 g/g of starch) after fermentation for 6 days. No latency phase was observed; microorganism growth occurred during the first 12 h of fermentation with a 1.1-h generation time. During this phase, the bacterial population changed from 3×10^5 to 1.5×10^{10} bacteria per ml and subsequently remained stable until the end of fermentation. Production of amylase, which was found in the culture supernatant, occurred at the end of the growth phase (90% of the amylase activity was formed during the first 8 h of the stationary phase). The cells then functioned as resting cells and continued to produce lactic acid until exhaustion of the substrate.

Fermentation of *L. plantarum* A6 on raw cassava starch. The inoculum size used in the trials was increased from 0.01 to 10% to achieve development of *L. plantarum* A6. Two parallel fermentation were performed, one with controlled pH (Fig.3) and one without pH control (Fig. 4). Although the starch had not been sterilized, no development of contaminant microorganisms was observed under optical microscopy, and HPLC did not reveal the production of any metabolite other than lactic acid. In both cases, the growth phase of *L. plantarum* A6 was short, being completed during the first 6 h. The final populations attained comparable levels. However, the differences in amylase activity were quite significant. Maximum amylase activity, 60 U/ml, was obtained with a controlled pH as compared with amylase activity of 2 U/ml with noncontrolled pH. It appeared that the pH should be kept at about 6.0 for bacterial cells to produce sufficient amylase activity to ensure the complete hydrolysis of raw starch granules. As before, amylase activity was produced principally at the end of the growing phase, enabling full conversion of raw starch granules to lactic acid. This gave 41 g of lactic acid from 45 g of raw starch after 3 days of fermentation. In fermentation without pH control, the culture pH rapidly fell below a level that the organism could tolerate. No amylase synthesis occurred during the stationary phase, and no noteworthy disappearance of starch was observed. Fermentation stopped rapidly, and the quantity of lactic acid produced was limited at 8 g/liter after the first day of fermentation.

SEM observation of digested starch granules. Enzymatic attack of raw starch during fermentation with controlled pH was observed by using SEM. The initially smooth granules (Fig. 1B) were rougher after 24 h of fermentation (Fig. 1C), and some displayed large cavities (Fig. 1D). Shell residues resulting from total digestion of the inner part of granules were observed (Fig. 1E). Enzymatic hydrolysis also revealed the lamellar organization of the starch granules (Fig. 1F). However, the progress of degradation was not homogeneous. Smooth granules and entirely digested granules were observed in the same sample. In addition, the proportion of granules displaying enzymatic attack appeared to be fairly small, whatever the stage of fermentation. A decrease in the number of granules was observed as fermentation progressed, with total disappearance occurring after 3 days. No strongly degraded granules and only slight surface erosion of some granules were observed in fermentation without pH control.

DISCUSSION

Numerous bacteria have been described in the literature as being amylolytic, but very few are capable of breaking down raw starch. Dettori-Campus et al. (6) sought the property in over 80 *Bacillus* strains and observed that only strains of *B. stearothermophilus* and *Bacillus amylolyticus* effectively degraded raw starch. Amylase synthesis is a rare characteristic of lactic acid bacteria. The principal strains of this type to be identified are *Streptococcus bovis*, *Streptococcus equinus*, *Lactobacillus amylophilus*, *Lactobacillus amylovorus*, *Lactobacillus acidophilus*, and *Lactobacillus cellobiosus* (11, 14, 17, 18, 24). Others isolated from animal digestive tracts have been described as amylolytic (2, 3). The ability to break down raw starch has been shown only in *S. bovis* 148 isolated from bovine rumen (20) and in *L. amylovorus* isolated from cattle waste-corn fermentation (13). It is shown here for the first time that a wild strain of *L. plantarum* isolated from fermented cassava can break down cassava raw starch. It was also found that pH control was required to enable *L. plantarum* A6 to achieve the complete hydrolysis of raw starch granules.

Lactic acid production causing rapid acidification of the medium to pH 3.6 was observed during fermentation on raw starch without pH control. This resulted mainly from the conversion by the microorganism of low-molecular-weight sugars present initially in the cassava and probably also from the weak hydrolysis of starch granules. Surface erosion of certain granules was detected by SEM. This limiting of the degradation phenomenon is related, first, to the small amylase activity synthesized (1/30 of that with controlled pH) and, second, to the weak activity of the enzyme at such a pH (only 15% relative activity at pH 3.5 in comparison with 100% at pH 5.5) (9). The effect of the pH on amylase synthesis had already been observed for *L. plantarum* A6 in flask cultures (7); initial addition of calcium carbonate to limit acidification of the medium increased the amount of amylase synthesized fivefold. This effect was also reported by Pompeyo et al. (19) for *L. amylovorus* and *L. amylophilus*, where larger amounts of amylase were found in the presence of $CaCO_3$.

Total conversion of starch granules to lactic acid was ob-

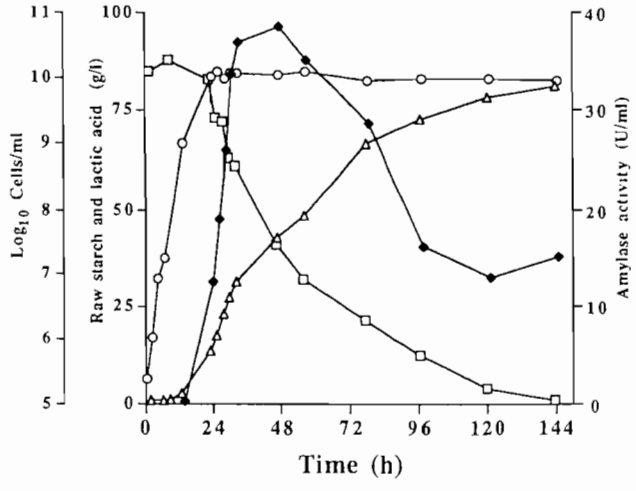

FIG. 2. Fermentation of *L. plantarum* A6 on dry-heated cassava starch at 30°C and pH 6.0. Symbols: □, starch; △, lactic acid; ◆, amylase activity; ○, $\log_{10}$ cells.

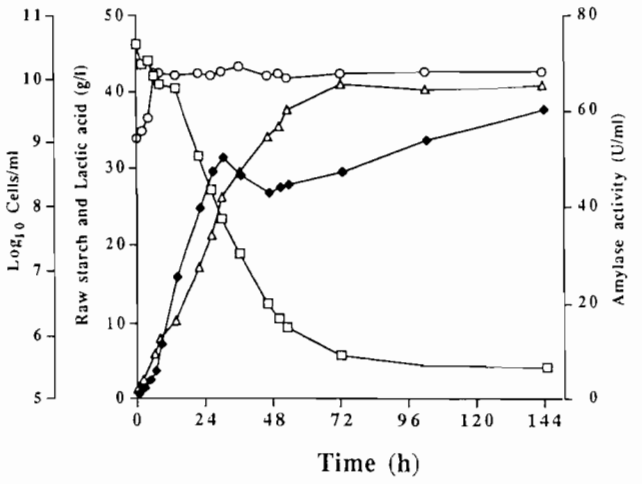

FIG. 3. Fermentation of *L. plantarum* A6 on raw cassava starch at 30°C and pH 6.0. Symbols: □, starch; △, lactic acid; ◆, amylase activity; ○, $\log_{10}$ cells

served for both dry-heated starch and raw starch during fermentation with pH control. The present results showed that amylase activity was not associated with cell growth, in contrast with previous work (8) in which it was found that enzyme production more closely paralleled growth of the culture. The difference may be due to substrate quality: cassava raw starch was used here instead of the soluble starch of previous experiments or the medium composition. Further studies would be required to clarify this point.

The results indicate that mass inoculation of certain natural fermentation processes with *L. plantarum* A6 will not cause total hydrolysis but simply a weak attack of the starch granules. This was observed in the case of natural cassava fermentation for production of gari, a traditional foodstuff in West Africa. The use of *L. plantarum* A6 resulted in faster acidification and a higher lactic acid content (4 g per 100 g [dry weight]), i.e., an increase of over 25% in comparison with results with a nonamylolytic strain of *L. plantarum* (10).

These encouraging results make it possible to envisage numerous applications of *L. plantarum* A6 as a starter in some traditional food fermentation processes where the quantity of lactic acid produced is sometimes too small to obtain a high-quality product. In addition, the capacity of *L. plantarum* A6 to fully convert starch into lactic acid when fermentation conditions are controlled might be of considerable interest for industrial production of the acid directly from raw starch, especially if a mutant derived from this strain is found to produce nonracemic lactic acid.

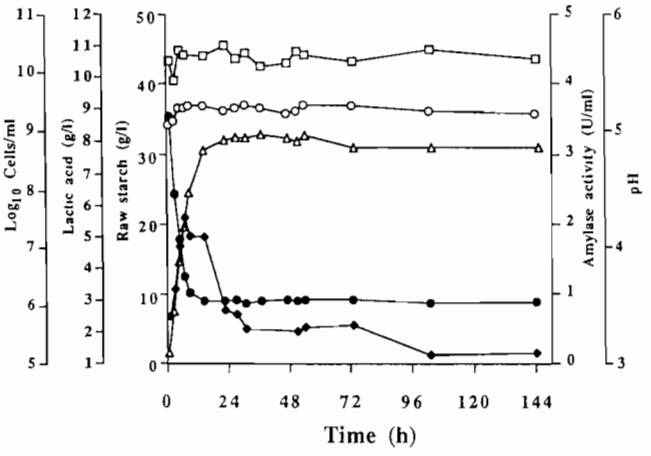

FIG. 4. Fermentation of *L. plantarum* A6 on raw cassava starch at 30°C without pH control. Symbols: □, starch; △, lactic acid; ◆, amylase activity; ○, $\log_{10}$ cells; ●, pH.

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ARTICLE 8

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Molecular characterization of the α-amylase genes of *Lactobacillus plantarum* A6 and *Lactobacillus amylovorus* reveals an unusual 3' end structure with direct tandem repeats and suggests a common evolutionary origin.

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Molecular characterization of the α-amylase genes of *Lactobacillus plantarum* A6 and *Lactobacillus amylovorus* reveals an unusual 3′ end structure with direct tandem repeats and suggests a common evolutionary origin

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Abstract

The α-amylase gene (amy*A*) *of Lactobacillus plantarum* A6 was isolated from the genome by polymerase chain reaction with degenerated oligonucleotides, synthesized according to the tryptic peptide amino acid sequences of the purified enzyme. Nucleic acid sequence analysis revealed one open reading frame of 2739 bp encoding a 913 amino acid protein. The amylase appears to be divided into two equal parts. The N-terminal part has the typical characteristics of the well-known α-amylase family (65% identity with the α-amylase of *Bacillus subtilis* and 97% identity with the partial sequence available for the α-amylase of *Lactobacillus amylovorus*). The C-terminal part displays a fairly unusual structure. It consists of four direct tandem repeated sequences of 104 amino acids sharing 100% similarity. The complete nucleotide sequence of the α-amylase gene of *L. amylovorus* was also determined. An open reading frame of 2862 bp encoding a 954 amino acid protein was identified. Perfect homology between the two *amy*A genes was observed in the N-terminal region. The C-terminal part of *L. amylovorus* α-amylase also included tandem repeat units but striking differences were observed: (i) the addition of one repeat unit; (ii) a shorter, 91 amino acid repetition unit. These structural homologies suggest that both genes have a common ancestor and may have evolved independently by duplication with subsequent recombination and mutation. © 1997 Elsevier Science B.V.

Keywords: Lactic acid bacteria; Gene organization; Repeated sequences; Evolutionary origin

1. Introduction

Lactobacillus plantarum is recommended as starter culture for fermentation controls of silage, cabbage, cucumber, olive and cassava (Vaughn, 1985; Seale, 1986; Daeschel and Fleming, 1987). Silage sometimes contains too little fermentable sugar to ensure the rapid production of a stabilizing amount of lactic acid. To overcome this difficulty, attempts have been made in recent years to produce a recombined strain of *L. plantarum* releasing a high amylolytic activity by the introduction of the α-amylase gene of *Bacillus amyloliquefaciens* (Jones and Warner, 1990) or *Bacillus stearothermophilus* (Scheirlinck et al., 1989). Nevertheless, the amount of α-amylase excreted by such recombinants was negligible. Recently, Fitzsimons et al. (1994) developed an amylolytic *L. plantarum* strain by the insertion in the chromosome of an active fragment of the gene encoding α-amylase production in *L. amylovorus*. The recombined *L. plantarum* strain was able to achieve amylolytic activity at a level similar to that of *L. amylovorus*. However, the use of such a genetically engineered strain under natural fermentation conditions may raise legal or ecological problems.

A wild strain of *L. plantarum* (strain A6) was previously isolated from retted cassava and selected for its ability to break down soluble starch (Giraud et al., 1991). The characteristics of this strain are particularly attractive. An amylolytic *L. plantarum* strain was reported for the first time. Moreover, it was found to

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synthesize large amounts of α-amylase. It was recently shown that *L. plantarum* A6 can also break down cassava raw starch without any preliminary physico-chemical treatment (Giraud et al., 1994). New applications of *L. plantarum* A6 might result from these promising results. It could be used as a starter when traditional fermentation processes of starch-containing food fail to produce sufficient lactic acid.

Molecular study of the amylase gene (*amy*A) of *L. plantarum* A6 was used to follow the development and the dispersion of the microorganism during traditional fermentation processes and to understand the origin of its unusual property.

Many microbial and eukaryotic α-amylase genes have been cloned and characterized (Horii et al., 1987; Korman et al., 1990; Sutliff et al., 1991; Rumbak et al., 1991). However, little work has been performed on the α-amylase genes of lactic acid bacteria. Only the α-amylase gene of *Streptococcus bovis* has been completely sequenced (Cotta and Whitehead, 1993), and the nucleotide sequence of the 5′ end of the *L. amylovorus* amylase gene has been determined (Fitzsimons et al., 1994). No significant homology has been found between these two genes.

The cloning and sequencing of the α-amylase gene of *L. plantarum* A6 are described here and the unusual structure of the 3′ end of this gene is shown. Furthermore, as substantial homology was observed with the 5′ end of the *L. amylovorus* amylase gene (>97%), the latter was fully characterized to determine the extent of the homology.

2. Materials and methods

2.1. Bacterial strains and growth media

L. plantarum A6 was isolated from retted cassava (Giraud et al., 1991) and *L. amylovorus* CIP 102989 was purchased from Institut Pasteur (France). For cloning experiments, *Escherichia coli* XL1-blue was used as a host strain. MRS (Difco Laboratories, Detroit, MI, USA) and LB (GIBCO) media were used for routine culturing of lactobacilli and *E. coli*, respectively.

2.2. Purification of the α-amylase and amino acid sequencing

α-Amylase of *L. plantarum* A6 was purified as described previously (Giraud et al., 1993). An aliquot of the protein (approx. 300 pmoles) was treated with 0.5 μg trypsin in 0.1 M Tris buffer (pH 8.1) overnight at 37°C. The resulting digest was fractionated by reverse-phase HPLC on a Brownlee C_8, 2 × 100 mm column (Perkin Elmer, Foster City, CA, USA) and eluted by a linear acetonitrile gradient in 0.1% trifluoracetic acid. The eluate was monitored by UV densitometry (220 nm). The resulting peptides were further purified using a Brownlee C_{18} column with the same experimental conditions. Selected peptides were submitted to Edman N-terminal sequential analysis in a 470-A protein sequencer connected on-line with a 120-A PTH analyser (Perkin Elmer). Both were operated according to the manufacturers' recommendations.

2.3. Southern hybridization analyses

L. plantarum A6 genomic DNA prepared as described by Gasson and Davies (1980) was digested with restriction endonucleases, separated by agarose gel electrophoresis and transferred to nylon membranes (Hybond-N$^+$; Amersham, Amersham, UK) using standard methods (Sambrook et al., 1989). DNA probes were labelled with digoxigenin (DIG)-dUTP using the DIG labelling kit supplied by Boehringer-Mannheim (Mannheim, Germany). Hybridization and detection experiments were performed with a DIG detection kit (Boehringer-Mannheim).

2.4. Amylase gene cloning

Total DNA was digested to completion by *Hin*pI and size-fractionated on 1% agarose gel. The 2.7 kb fragments bearing the α-amylase gene were eluted from the gel and ligated into the *Acc*I site of plasmid pGEM-3Zf(+) (Promega, Madison, WI, USA). Transformation of *E. coli* electro-competent cells was performed with a Bio-Rad Gene Pulser unit (Bio-Rad, Hercules, CA, USA). After replica plating, the positive clones containing the *amy*A gene were screened from the gene libraries by colony hybridization under the same conditions as those used for southern hybridization analyses.

2.5. DNA amplification and cloning of PCR fragments

PCR reactions were performed in 50 μl final volume containing: 0.1–0.5 μg of DNA and deoxyribonucleoside triphosphate (200 μM each), primers (0.8 μM each), $MgCl_2$ (1.5 mM), 1.25 unit *Taq* DNA polymerase (Promega) and the buffer supplied with the enzyme. The amplification conditions were: initial denaturation at 94°C for 5 min, 30 cycles with a 30 s denaturation step at 94°C, 30 s annealing step at 50 or 55°C (according to the T_m of primers used) and an elongation step at 72°C lasting 1–3 min (depending on the length of fragment to be generated). After the final elongation step at 72°C for 7 min, the amplified fragments were purified by a Wizard procedure and ligated into pGEM-T vector (Promega).

2.6. Sequencing of the DNA

Both strands of the inserts of the different clones were sequenced by the dideoxy-chain termination method (Sanger et al., 1977) with an 373 DNA Sequencer (Applied Biosystems, Foster City, CA, USA). Protein homology searches were carried out with the SWISS-PROT (release 34) database and genomic sequences with the GenBank (release 98) database and the FASTA program (Pearson and Lipman, 1988).

2.7. Nucleotide sequence accession numbers

The GenBank accession numbers for *L. plantarum* A6 and *L. amylovorus* α-amylase genes are U62095 and U62096, respectively.

3. Results

3.1. Amplification of a specific probe of α-amylase gene of L plantarum

Purified α-amylase of *L. plantarum* A6 (Giraud et al., 1993) was subjected to trypsin digestion and the resulting peptides were separated by reversed-phase chromatography. Three peptides were selected for sequencing analysis, and the sequences (Table 1) were compared with those of the protein data bank (SWISS-PROT). Strong homology was found for the three peptides with the sequence of the α-amylase of *Bacillus subtilis* (Yang et al., 1983). The degenerate primers A and B were then designed from peptide 1 (amino acids DWTHGNT) and peptide 2 (amino acids WPNITDN). The marked homology found with the α-amylase of *B. subtilis* led us to define primer C (amino acids EFKEMCA) corresponding to the less degenerated amino acid region of the AMYA of *B. subtilis*.

Primers A–B led to the amplification of a 253-bp fragment and primers B–C to the amplification of a 371-bp fragment from the genomic DNA of *L. plantarum* A6. Their sequences revealed a high identity level (65%) with the α-amylase gene of *B. subtilis*.

3.2. Isolation of the L. plantarum A6 amyA gene

Southern hybridization analyses with the 371-bp DIG-labelled fragment as a probe was performed (Fig. 1). Two bands were observed, except for *Pst*I digestion. The variation range (600–700 bp) between the two bands was similar for all the digestions. Since the enzyme sites used in this analysis were not present in the probe (except for *Hin*dI, whose site is located at the 5′ end of the probe) and since no hybridization signal was observed with the plasmidic fraction of *L. plantarum* A6 DNA (data not shown), these results suggest that *L. plantarum* A6 contains at least two chromosomal *amy*A gene copies.

A first attempt was made at cloning the entire gene. The 5.2-kb *Hin*dIII fragments were eluted from an agarose gel and ligated into the dephosphorylated *Hin*dIII site of vector pGEM-3Zf(+). No hybridization signal was observed after screening 1200 recombinant clones. These results suggest that the entire amylase gene or another region of the chromosome flanking the amylase gene was lethal in *E. coli*, as has already been observed for *B. subtilis amyA* gene cloning (Yang et al., 1983; Emori et al., 1990).

It was therefore decided to isolate the gene in truncated segments. The 2.7-kb *Hin*dI fragment pool was isolated from the gel, and cloned into the vector pGEM-3Zf(+) linearized with *Acc*I. Of the 500 recombinant clones, five hybridized with the 371-bp DIG-labelled fragment, one of these was then sequenced.

The 2.7-kb fragment corresponds to approximately 80% of the DNA that encodes the mature part of the α-amylase protein but the 5′ end of the entire gene is lacking. This 5′ end was then obtained using a PCR screening plasmid approach. The 5.2-kb *Hin*dIII fragment pool was isolated from a gel and ligated into a pGEM-3Zf(+) vector linearized with *Hin*dIII. A primer deduced from the *Hin*dI fragment sequence and the universal oligonucleotides (M13 forward and M13 reverse) of the vector plasmid were used to amplified a 1 kb fragment corresponding to the lacking region of the *amy*A gene.

Table 1
Peptide sequences of the α-amylase of *L. plantarum* A6 and their homology with the α-amylase of *B. subtilis*

Peptide No	Sequence	Homology with AMYA of *B subtilis* (amino acid location)
1	SISDWTHGNTQISNWSDR	SISD WTHGNTQISN WSDR II · IIIIIIII I IIII 151 ISNEVKSIPN WTHGNTQIKN WSDRWDVTQN 181
2	HIELPSQYDGSYGSNFWPNITDNG	HIELPTQYDG SYGSNFWPNI TDNG IIIII: II IIII:IIIII I . 221 HIELPD--DG SYGSQFWPNI TNTSAEFQYGEI 251
3	LGWAVVASR	LG WAVVASR II III:III 321 WMSDDDIRLG WAVIASRSGS 341

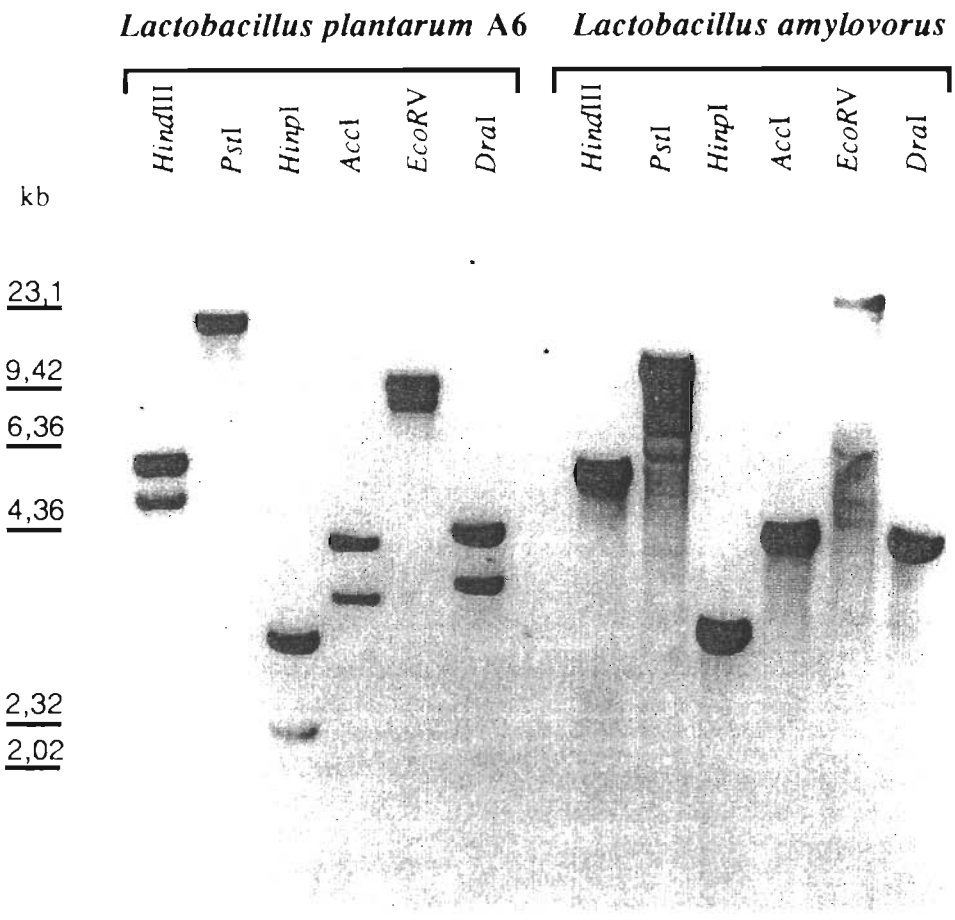

Fig. 1. Southern hybridization analyses of the α-amylase genes of *L. plantarum* A6 and *L. amylovorus*. Chromosomal DNA of *L. plantarum* A6 and *L. amylovorus* digested with *Hin*dIII, *Pst*I, *Hin*pI, *Acc*I, *Eco*RV and *Dra*I was subjected to electrophoresis on 1% agarose gel and blotted on nylon membranes. The 371-bp DIG-labelled DNA (fragment B–C) was used as a probe.

3.3. *Nucleotide sequence of L. plantarum A6* amy*A gene*

An open reading frame (*amy*A) of 2739 bp encoding a 913 amino acid protein with a molecular mass of 99 544 Da was identified. Fig. 2 shows that the open reading frame starts with a GTG codon at nucleotide position 363, which is preceded at a canonical distance (9 nucleotides) by a putative ribosome-binding site (AAAGGGGG) complementary to the 3′ end of the *L. plantarum* 16S rRNA (UCUUUCCUCCAC; complementary bases underlined) (Woese et al., 1992, unpublished).

The first 108 nucleotides of the structural gene encode a characteristic Gram-positive signal peptide of 36 amino acids (von Heijne, 1985). The entire protein has a predicted theoretical isoelectric point (pI) of 4.63. The amylase gene appears to be divided into two main regions, joined at the *Bam*HI site. The first 5′ half, corresponding to the first 480 amino acids, revealed typical features of the well-characterized α-amylase family (Rogers, 1985). The greatest similarity was found with a limited number of α-amylases: (i) 96.8% identity in 155 amino acids overlaps with the partial sequence of α-amylase from *L. amylovorus* (Fitzsimons et al., 1994); (ii) 64.5% identity in 462 amino acids overlaps with the α-amylase of *B. subtilis* (Yang et al., 1983); (iii) 27.2% identity in 430 amino acids overlaps with the α-amylase of *Butyrivibrio fibrisolvens* (Rumbak et al., 1991).

The 3′ half displays an unusual structure consisting of tandem repeated sequences; it consists of four perfectly homologous 104 amino acid repeats, each of them beginning with a microsatellite-like structure (agc agc agc agt agt agt aca aca aca). This microsatellite-like structure has been well documented in eukaryotic genomes (Tautz and Renz, 1984; Bruford and Wayne, 1993), but, to the best of our knowledge, is extremely rare in prokaryotic genomes. Furthermore, a perfectly repeated octanucleotide sequence caagcagc is found at the borders of each repeat, suggesting an insertion event followed by duplication in an ancestral α-amylase gene.

This repeated region was used as a probe in Southern hybridization analyses. The same pattern as for the 371-bp probe (data not shown) was observed, indicating

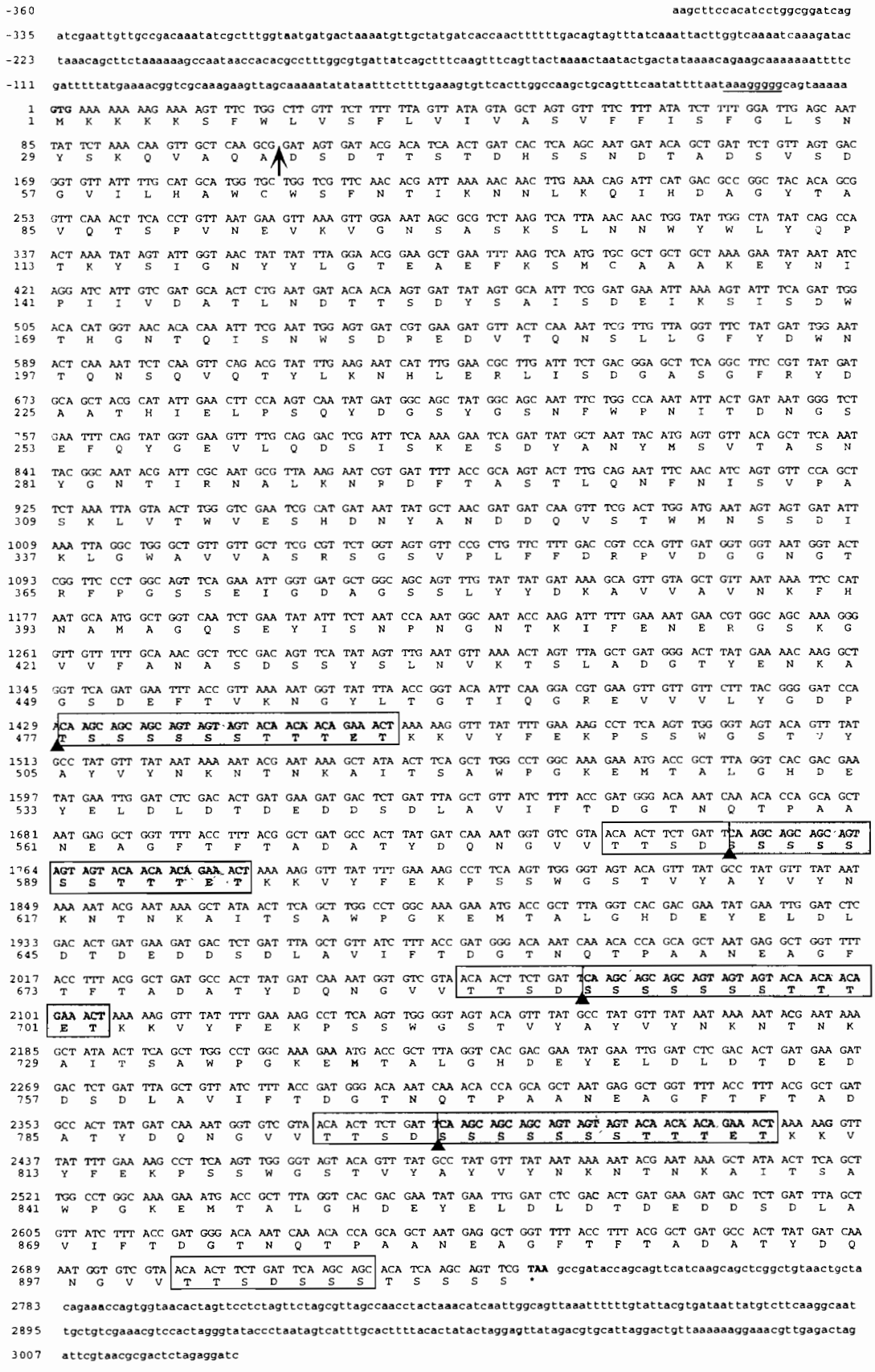

Fig. 2. The nucleotide sequence and the deduced amino acid sequence of the *amy*A of *L plantarum* A6. Putative ribosome-binding sites (underlined), start and stop codon (boldface) are indicated. The presumed signal peptidase cleavage site is indicated with an arrow. The beginning of each repeated sequence is highlighted with a triangle. The 5′-end flanking regions of the 'consensus' repeat unit are shaded and the 3′-end flanking regions of the 'consensus' repeat unit are boxed.

that these repeats are always associated with α-amylase genes and that other similar repeats are not dispersed in the chromosome.

3.4. *Nucleotide sequence of the* L. amylovorus amyA *gene*

The strong homology between the partially sequenced α-amylase gene of *L. amylovorus* and the corresponding region in *L. plantarum* A6 led us to perform full characterization of the former

Southern hybridization analyses of the *L. amylovorus* chromosomal DNA were carried out using the same restriction enzymes and the same probes as for *L. plantarum*. A main band was observed for each digest (Fig. 1) suggesting that the *amy*A gene is unique in *L. amylovorus*. It is interesting to note that four out of six digests yield very similar fragment sizes in the two different species. Thus, it was decided to use the primers defined for the sequencing of the *amy*A gene of *L. plantarum* A6 to isolate and sequence the *amy*A gene of *L amylovorus*.

A 2862 bp long open reading frame (*amy*A) encoding a protein of 954 amino acids with a molecular mass of 104 674 Da was identified (Fig. 3). Perfect homology between the two *amy*A genes can be observed in the first 5′ half (up to the *Bam*HI site) corresponding to the active site, with only seven mismatches in 1600 nucleotides.

As for *L. plantarum* A6 *amy*A, the second part shows the presence of tandem repeat units but striking differences are observed (Fig. 4): (i) Five repeat units are present in *L. amylovorus* as opposed to four in *L. plantarum*. (ii) The repetition unit is shorter (273 nucleotides instead of 321). These repeat units encode 91 amino acids and are homologous with each other and show only five mutations with the corresponding part of the *L. plantarum* A6 repeat unit.

The comparison of repeated sequences of the two *amy*A genes led us to define a 'consensus' repeat unit of 273 nucleotides bordered by two regions: (i) a 5′ end flanking region of 35 nucleotides containing the octanucleotide insertion site and the microsatellite-like structure; (ii) a 3′ end flanking region of 21 nucleotides containing the octanucleotide insertion site. These flanking regions are found at the extremity of each repeat unit of 273 nucleotides for *L. plantarum* A6, but only once at the borders of the tandem repeated region of *L. amylovorus* (Fig. 2, Fig. 3 and Fig. 4). Note the deletion of the two trinucleotides agt in the microsatellite-like structure of *L. amylovorus*.

The particular structure of the *amy*A genes and the comparison of the tandem repeats suggest the insertion of a 321 nucleotides sequence in an ancestral gene of α-amylase, followed by dispersion in the two *Lactobacillus* species with duplications specific to each strain.

4. Discussion

The molecular characterization of the *amy*A genes of *L. plantarum* A6 and *L. amylovorus* has been described. A very high homology between the two genes is observed, raising the question of the correct identification of the A6 strain. We have sequenced a highly variable region corresponding to the first 400 bp of the 16S rRNA of the A6 strain, and have found a perfect similarity with the *L. plantarum* 16S rRNA sequence (Woese et al., 1992, unpublished). This last result confirms without any ambiguity the identification of the A6 strain to the species *L. plantarum*.

The amino acid sequences deduced indicate that both *amy*A genes encode a large polypeptide with a molecular weight of about 100 kDa. These polypeptides are approximately twice as large as typical microbial α-amylases (50–60 kDa) (Vihinen and Mäntsälä, 1989). However, they are smaller than the estimated molecular weights of the corresponding purified proteins. The amylase protein of *L. amylovorus* has been estimated to be 126 kDa (Jore and DeParasis, 1993) or 150 kDa (Iman et al., 1991). SDS-PAGE analysis of the purified α-amylase of *L. plantarum* A6 resulted in a well-defined band (50 kDa) and a diffuse band close to 150 kDa (Giraud et al., 1993). The discrepancies in the estimation of the size can been explained either by migration artefacts of the proteins in SDS-PAGE, as has been reported for some amylases (Robyt and Ackerman, 1973; Arakawa et al., 1992), or by glycosylation of the protein, as observed for bacterial amylases such as those of *Alicyclobacillus acidocaldarius* (Schwermann et al., 1994), or *Bacillus brevis* (Stefanova and Emanuilova, 1992).

A remarkable feature of the structure of both *amy*A genes is the presence, at the 3′ end, of direct tandem repeat sequences which share 100% similarity with each other. A striking difference is that the repetition number and the size of repeats vary between the two organisms: four repeated sequences of 321 nucleotides for the *amy*A of *L. plantarum* A6, five repeated sequences of 273 nucleotides for *L. amylovorus*. Southern hybridization analysis showed another noticeable difference between the strains. Whereas only one copy of the *amy*A gene is observed for *L. amylovorus*, two copies were revealed in *L. plantarum* A6. For all the digestion, except for *Pst*I, the size differences between the two copies were always in the same range (600–700 bp). It can be suggested that both gene copies observed in *L. plantarum* A6 differ by only two 321-bp repeat units and that these duplicated *amy*-genes are located within a single large *Pst*I fragment.

We have investigated by PCR experiments the presence of such repeated sequences in other lactic acid bacteria (*Lactococcus lactis* ATCC 11454, *Leuconostoc mesenteroides* ATCC10832, *Pediococcus pentosaceus*

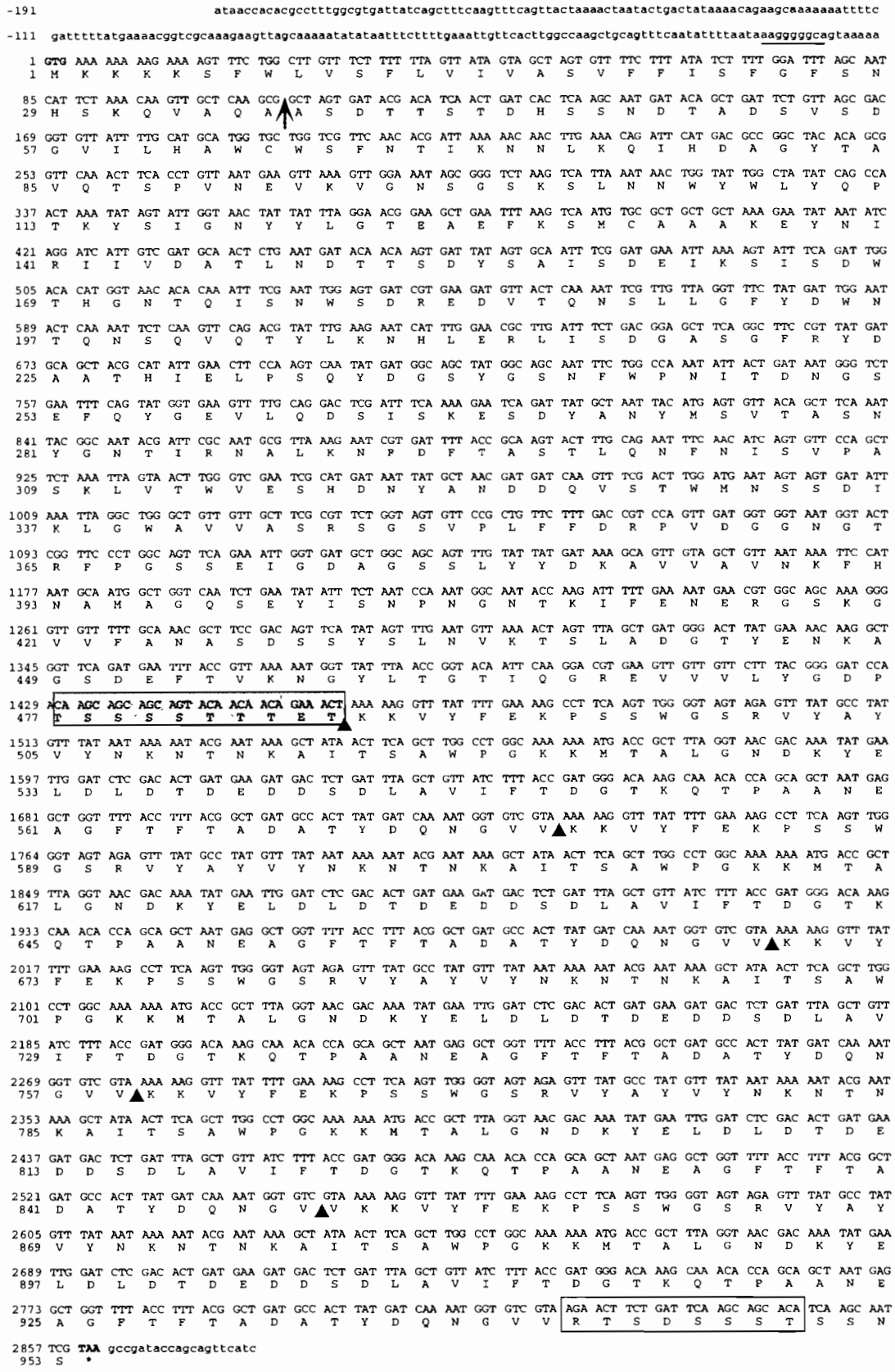

Fig. 3. The nucleotide sequence and the deduced amino acid sequence of the *amy*A of *L. amylovorus*. Putative ribosome-binding sites (underlined), start and stop codon (boldface) are indicated. The presumed signal peptidase cleavage site is indicated by an arrow. The beginning of each repeated sequence is highlighted with a triangle. The 5′-end flanking region of the 'consensus' repeat unit is shaded and the 3′-end flanking region of the 'consensus' repeat unit is boxed.

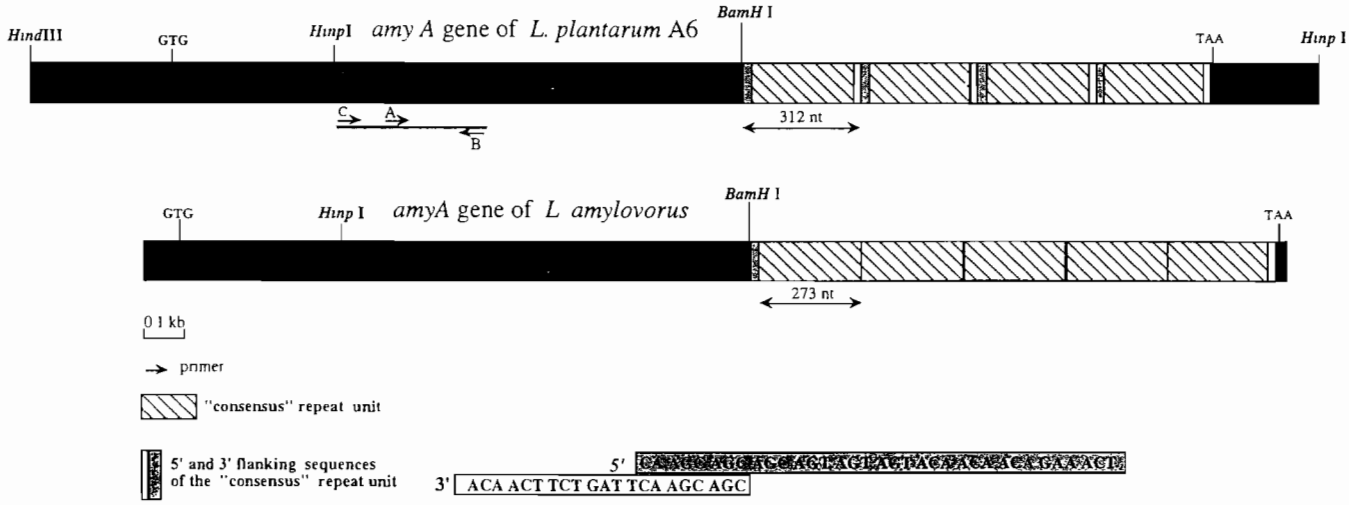

Fig 4. Diagrammatic sketch of the α-amylase genes of *L plantarum* A6 and *L amylovorus*.

ATCC 43200) and in other *L. plantarum* strains (ATCC 14917, ATCC 10241 and DSM 20174). No amplification products were obtained for all the strains tested; these repeat sequences appear specific to *L. plantarum* A6 and *L amylovorus*.

Jore and DeParasis (1993), using a series of deletion derivatives in the 3′ end of the α-amylase gene of *L. amylovorus*, reported that the N-terminal part (45 kDa) still displays full α-amylase activity. Likewise, Fitzsimons et al. (1994) have demonstrated that the first 590 nucleotides of the *L. amylovorus amy*A gene, including the promoter region, are sufficient to transfer an amylolytic activity to a strain of *L. plantarum* which naturally lacks such activity. The fact that more than 50% of the C-terminal part of the α-amylase is not essential for amylolytic activity raises the question of the function of this region.

The amylases from *B. subtilis*, *L. amylovorus*, and *L. plantarum* A6 have identical physico-chemical properties (same optimal pH and optimal temperature) (Welker and Campbell, 1967; Fogarty, 1983; Giraud et al., 1993; Pompeyo et al., 1993). Nevertheless, only *L. plantarum* A6 and *L. amylovorus* possess the special ability to break down raw starch (Giraud et al., 1994; Iman et al., 1991). It is therefore tempting to suggest that the particular structure of the C-terminal half of the α-amylases of these two strains is associated with effective substrate binding. This hypothesis is supported by the description of tandem repeat unit located in the C-terminal portion of a family of clostridial and streptococcal ligand-binding proteins (Wren, 1991). This group of proteins includes toxins from *Clostridium difficile* (von Eichel-Streiber et al., 1992), glycosyltransferases from *Streptococcus* strains (Giffard and Jacques, 1994) and toxin from *Streptococcus pneumoniae* (Yother and Briles, 1992). This family of ligand-binding proteins appears to display a modular design, with one module providing enzymatic functions and the other module consisting of a repetitive carbohydrate-binding domain located in the C-terminal region (Wren, 1991).

The high degree of homology in the DNA sequences for the two α-amylase genes suggests that these genes are evolutionary related to each other. The structural homologies, particularly in the 3′ end part, indicate that both genes probably have a common ancestor and may have evolved independently by duplication and subsequent recombination and mutation, as has been reported for streptococcal glycosyltransferases (Wren, 1991; Giffard and Jacques, 1994) and for *Clostridium difficile* toxins (von Eichel-Streiber et al., 1992). The significant phylogenetic distance between the two *Lactobacillus* strains (Collins et al., 1991) led us to consider that the acquisition of this unusual amylolytic property in *L. plantarum* A6 results from a lateral transfer of this ancestor common gene. Since the repeated sequences are perfectly homologous in one strain and quite well preserved between the two strains, it can be assumed that such an event occurred relatively recently.

The role of these repeated sequences remains to be elucidated. The expression of the entire gene and/or 3′-end truncated forms may confirm their importance in raw starch binding.

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ARTICLE 9

Giraud E, Champailler A, Moulard S et Raimbault M.

Development of a miniaturised selective strategy of lactic acid bacteria for evaluation of mixed starter in a model cassava fermentation.

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Development of a miniaturized selective counting strategy of lactic acid bacteria for evaluation of mixed starter in a model cassava fermentation

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E GIRAUD, A. CHAMPAILLER, S MOULARD AND M RAIMBAULT 1998 A miniaturized most probable number (MPN) method for the selective enumeration of three bacteria species (*Lactobacillus plantarum* A6, *Leuconostoc mesenteroides* and *Lactococcus lactis*) is described. This selective count method, based on specific consumption of carbon substrate and resistance to antibiotics, was used for the quantitative assessment of the three bacteria during mixed cultures in a model cassava fermentation. A typical microbial succession pattern was observed: (i) *Lactococcus lactis* and *Leuc. mesenteroides* dominated during the first hours of fermentation as their growth was very rapid; (ii) from hour 12, *Lactobacillus plantarum* replaced the two latter strains and *Lactococcus lactis* disappeared gradually, followed by *Leuc. mesenteroides*. The growth rates of each strain appeared to be independent of the others, while acidification rates increased strongly in mixed cultures compared with pure cultures. No positive interactions resulting from the amylolytic character of *Lactobacillus plantarum* A6, and no negative interactions resulting from the Nis$^+$ property of *Lactococcus lactis*, were revealed between the three strains under the model conditions used.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is the staple food of more than 500 million people (Cock 1982) and is a typical crop in developing countries. Cassava is considered to be a key component of famine prevention in African countries in spite of a certain degree of toxicity caused by the presence of cyanogenic glucosides (mainly linamarin) and the highly perishable nature of the roots after harvesting.

Native populations have empirically developed several procedures for stabilizing cassava and reducing its toxicity. Fermentation, which is part of almost all of these processes, is an important stage for obtaining the sensorial qualities required and for conservation (Ngaba and Lee 1979; Dougan *et al.* 1983). Most authors now agree that there is a predominant development of lactic acid bacteria during the various cassava natural fermentation processes (Okafor 1977; Ngaba and Lee 1979; Nwanko *et al.* 1989; Oyewele and Odunfa 1990; Brauman *et al.* 1996). However, as natural fermentations rely on microbial populations present in the raw material, they are subject to substantial variations in flavour and quality. This could be overcome by the use of a lactic acid bacteria starter to control fermentation and produce high quality products.

Initial investigations (Giraud *et al.* 1993a) designed to select a lactic acid bacteria starter for *gari* production enabled the isolation of *Lactobacillus plantarum* (strain A6) that possesses α-amylase and linamarase activities. However, the use of a single strain would seem too restrictive for the production of a foodstuff with a generous range of organoleptic characteristics. The use of a mixture of micro-organisms with complementary physiological and metabolic properties seems to be the best approach for obtaining a product with the nutritional and sanitary properties desired.

A novel selective enumeration strategy for the further investigation of the effects of a mixed starter on cassava fermentation is described here. Three lactic bacteria that regularly occur in natural plant fermentation were used as the model: (i) *Lactobacillus plantarum* A6, an amylolytic strain, (ii) *Leuconostoc mesenteroides* ssp. *mesenteroides*, and (iii) *Lactococcus lactis* ssp. *lactis*, a nisin-producing strain.

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MATERIALS AND METHODS

Organisms

The micro-organisms used were *Lactobacillus plantarum* strain A6 isolated from retted cassava (Giraud *et al.* 1991), Nip$^+$ *Lactococcus lactis* ssp. *lactis* ATTC 11454 and *Leuconostoc mesenteroides* ssp. *mesenteroides* ATCC 10880. The strains were conserved in glycerol (30%) at −80 °C

Medium and culture conditions

The basal liquid nutrient medium contained: 10 g soy peptone obtained by papain digestion, 0·5 g K_2HPO_4; 0·5 g KH_2PO_4, 0·2 g $MgSO_4.7H_2O$; 0·05 g $MnSO_4.H_2O$; 0·5 g $CaCl_2$ $2H_2O$ and 1000 ml distilled water The medium was sterilized at 121 °C for 20 min, cooled to about 30 °C and mixed with 100 g l^{-1} pre-sterilized cassava flour The latter was obtained from roots of cassava (*Manihot esculenta* var. Ngansa) harvested in the Brazzaville region 15 months after planting The roots were peeled, diced and frozen at −80 °C and freeze-dried for 48 h The flour obtained by grinding and sieving, with a 5% (w/w) moisture content, was autoclaved separately to avoid starch gelatinization (121 °C, 20 min) The media thus prepared were transferred to a previously autoclaved bioreactor and inoculated with one, two or three strains according to the experiment A 20 h pre-culture of each strain was performed on MRS (Difco Laboratories, Detroit, MI, USA) The bacteria were inoculated at 10^5 cells ml^{-1} for each strain The cultures were performed in a 2 l bioreactor (Biolafitte, Poissy, France) at 30 °C and agitated at 300 rev min^{-1} The initial pH was around 6, it was not regulated, but measured. Each culture experiment was duplicated.

Antibiotic resistance determination and carbohydrate utilization

Susceptibility of bacteria to antibiotics was examined by the antibiogramme kit, API ATB STREP strips (Biomérieux, Craponne, France; ref. 14050). Sample preparation and tests were performed according to the manufacturers' recommendations To confirm inhibition of growth by antibiotics, a selection of test cultures was performed on API 50CHL medium (ref. 50410; Biomérieux) containing glucose as substrate and selected antibiotics at 4 and 8 mg l^{-1}.

The bacterial test strains were also examined for their ability to utilize different carbohydrate sources by using the API 50 CH strips (ref 50300) according to the manufacturers' recommendations.

Estimation of the different bacterial populations

The bacterial populations were monitored using a miniaturized most probable number (MPN) method derived from that developed by Hernandez *et al.* (1991) and adapted for counting lactic bacteria. The culture medium was API 50CHL medium (ref 50410; Biomérieux). It was placed in 9 ml tubes and autoclaved (121 °C, 20 min). The carbon substrate (50 g l^{-1}) and the antibiotic (20 mg l^{-1}) specific to the micro-organism to be counted were sterilized separately by filtration (0·22 μm pore size, HAWP type; Millipore, Saint-Quentin-les-Yvelines, France) and distributed at 50 μl per well in sterile microtitration plates (96 wells) Three microtitration plates each containing the substrate and the antibiotic specific to each micro-organism were prepared in this way for each count. Decimal dilutions of the sample to be counted were performed directly in the API 50CHL medium and distributed at 200 μl per well with 12 wells per dilution on the previously prepared microplates. After incubation for 48 h at 30 °C, the wells in which the colour had changed from purple to yellow were counted as positive A computer program developed by Institut Pasteur, Lille, France (Hernandez *et al.* 1991) was then used to evaluate the most probable number of bacteria according to the number of positive wells

Analytical methods

Lactic acid, acetic acid and ethanol were determined in the supernatant fluid by HPLC using an Aminex HPX 87H column (Biorad Laboratories, Richmond, CA, USA) with a 0·8 ml min^{-1} flow of 0·006 mol l^{-1} H_2SO_4 at 65 °C and with refractive index detection

Assay of α-amylase activity

α-Amylase activity was assayed by observing degradation of starch by measurement of its iodine chelating ability. One enzyme unit is defined as the amount of enzyme that permits the hydrolysis of 10 mg of starch in 30 min under the conditions described by Giraud *et al.* (1993b).

Nisin assay

Culture supernatant fluid was heated in a microcentrifuge tube (1·5 ml) in a boiling water bath for 1 min and cooled rapidly on ice. Serial twofold dilutions of the heated supernatant fluid were performed in 0·02 mol l^{-1} HCl. The dilutions were examined for inhibitory activity by bioassay performed according to the quantitative agar diffusion method of Rogers and Montville (1991) using *Lactococcus lactis* ssp. *cremoris* ATCC 14365 as indicator organism. A standard curve (1–100 IU ml^{-1}) was plotted using a stock solution of 1000 IU ml^{-1}. The latter was prepared by dissolving 100 mg of Nisaplin (Sigma Chemical Co. Ltd, Saint Quentin Fallavier, France) to 100 ml 0·02 mol l^{-1} HCl and adjusting the pH to 5·3. Each assay was performed in triplicate.

RESULTS

Finalizing specific media for selective enumeration

A selective count strategy was developed to monitor the population dynamics of each micro-organism in mixed culture A search for specific features in the three bacteria selected was performed by comparing sugar utilization profiles and resistance to antibiotics The most relevant results are summarized in Table 1

These observations were used as the basis for complementary tests on microplates to verify satisfactory utilization of the carbon substrate and resistance to antibiotics for variable population levels (Table 2). It was observed that the utilization of melezitose and α-methyl-D-glucoside without antibiotic is specific to *Lactobacillus plantarum* A6 and *Leuc. mesenteroides*, respectively. It is noted that the addition of vancomycin to these media does not change the behaviour of these two strains. A specific count of *Lactococcus lactis* can only be obtained with the simultaneous presence of rifampicin and trimethoprim. Indeed, inhibition of the growth of *Lactobacillus plantarum* A6 was achieved only when these antibiotics were present, suggesting that rifampicin and trimethoprim have a synergetic effect on inhibition of this strain. The three following selective media were therefore chosen (i) API 50 CHL + melezitose + vancomycin for *Lactobacillus plantarum* A6; (ii) API 50 CHL + α-methyl-D-glucoside + vancomycin for *Leuc. mesenteroides*; (iii) API 50 CHL + glucose + rifampicin + trimethoprim for *Lactococcus lactis*.

Table 1 Carbohydrate utilization and antibiotic resistance of the three selected strains

	Lactobacillus plantarum A6	*Leuconostoc mesenteroides*	*Lactococcus lactis*
Melezitose 10 g l^{-1}	+	−	−
Starch 10 g l^{-1}	+	−	±
D-xylose 10 g l^{-1}	−	±	−
α-Methyl-D-glucoside 10 g l^{-1}	−	+	−
Tetracycline 8 mg l^{-1}	+	−	−
Vancomycin 4 mg l^{-1}	+	+	−
Trimethoprim 4 mg l^{-1}	±	±	+
Rifampicin 4 mg l^{-1}	±	−	+
Trimethoprim + Rifampicin	−	−	+

Interpretation of reactions −, negative; ±, weakly positive, +, positive For the antibiotic tests, glucose was used as carbohydrate substrate

Table 2 Effect of the inoculum level on specificity of carbohydrate utilization and antibiotic resistance for the three selected strains

	Lactobacillus plantarum A6			*Leuconostoc mesenteroides*			*Lactococcus lactis*		
Bacteria ml^{-1}	10^6	10^4	10^2	10^6	10^4	10^2	10^6	10^4	10^2
Glucose 10 g l^{-1}	+	+	+	+	+	+	+	+	+
Melezitose 10 g l^{-1}	+	+	+	−	−	−	−	−	−
α-Methyl-D-glucoside 10 g l^{-1}	−	−	−	+	+	+	−	−	−
Vancomycin 4 mg l^{-1}	+	+	+	+	+	+	−	−	−
Tetracycline 8 mg l^{-1}	+	−	−	−	−	−	−	−	−
Tetracycline 4 mg l^{-1}	+	±	−	−	−	−	−	−	−
Melezitose + Vancomycin	+	+	+	−	−	−	−	−	−
α-Methyl-D-glucoside + Vancomycin	−	−	−	+	+	+	−	−	−
Trimethoprim 4 mg l^{-1}	±	±	±	±	±	±	+	+	+
Rifampicin 4 mg l^{-1}	+	+	+	−	−	−	+	+	+
Trimethoprim + Rifampicin	−	−	−	−	−	−	+	+	+

Interpretation of reactions: +, total change in pH indicator; ±, partial change in pH indicator; −, no change in pH indicator. For the antibiotic tests, glucose was used as carbohydrate substrate when no other substrate is specified.

To validate this counting technique on microplates in the presence of the specific sugar and antibiotic, the growth of the strains in pure culture was monitored using three different methods: estimation of the population by counting the number of cfu on MRS agar medium; estimation of the population by determining the Most Probable Number on microplates using a non-selective medium (API 50CHL plus glucose) and the selective medium previously determined. The changes in the *Lactococcus lactis* population recorded in each of the three methods are shown in Fig 1 as an example It was observed that the total viable counts obtained were in agreement in the three methods and hence, for each micro-organism. A non-parametric analysis of variance according to the Kruskal–Wallis test (Zar 1984) was performed to compare the three methods; for the 11 times of sampling, the viable counts were not statistically different ($P > 0{\cdot}05$)

Pure cultures of lactic acid bacteria in a model cassava starch

Lactococcus lactis, *Leuc. mesenteroides* and *Lactobacillus plantarum* A6 were cultivated in fermenters without pH regulation on a cassava based sterile medium (Fig 2) The main fermentation parameters are shown in Table 3 The three strains grew rapidly with no latent phase; the maximum population level (more than 10^9 bacteria ml^{-1}) was observed after 12 h of fermentation.

Lactococcus lactis displayed the highest growth rate and the highest acidification rate. However, this population level did not remain stable and decreased from the second day of fermentation. No nisin production was detected on this medium. In contrast, on MRS under the same culture conditions, the strain produced concentrations of approximately 500 IU ml^{-1} (data not shown).

Leuconostoc mesenteroides displayed a relatively low acidi-

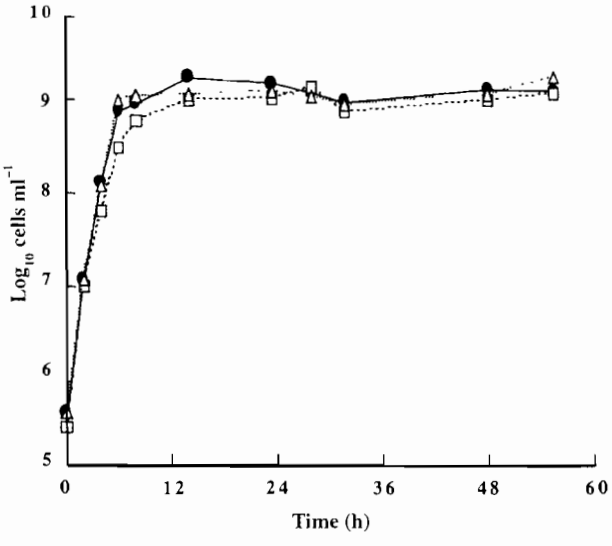

Fig. 1 Evaluation of the changes in a population of *Lactococcus lactis* in pure culture on cassava using three counting methods. □, MRS agar plate count method; ●, MPN method using API50 CHL + glucose; △, MPN method using API50 CHL + glucose + rifampicin + trimethoprim

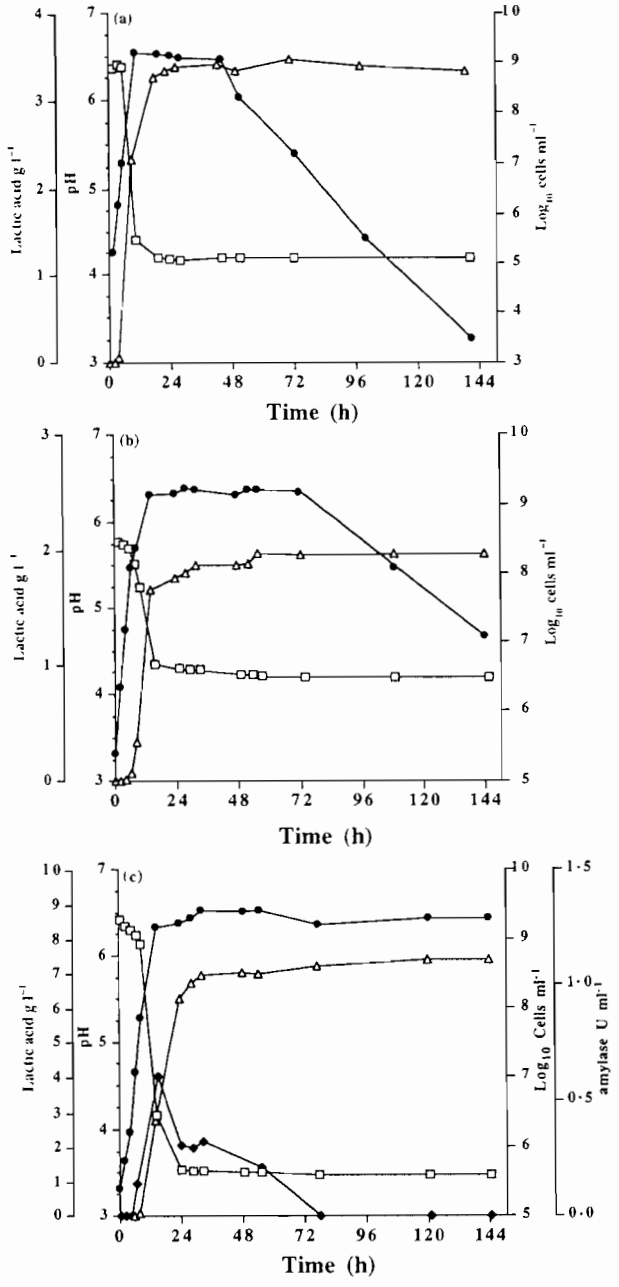

Fig. 2 Batch fermentation profiles of *Lactococcus lactis* (a), *Leuc. mesenteroides* (b) and *Lactobacillus plantarum* (c) cultivated in pure culture on a cassava-based sterile medium at 30 °C without pH control: △, Lactic acid, ●, Cells, □, pH; ◆, amylase activity

Table 3 Fermentative parameters of pure cultures

Micro-organism	Min pH	Maximal acidification rate (Δ pH/h)	Lactate g l^{-1}	Acetate g l^{-1}	Ethanol g l^{-1}	μ (h^{-1})	Maximal population cells ml^{-1}
Lactobacillus plantarum A6	3·47	0·33	7·4	0·7	—	0·65	$2·5\ 10^9$
Lactococcus lactis	4·20	0·39	3·5	0·1	—	1·05	$1·6\ 10^9$
Leuconostoc mesenteroides	4·20	0·16	2·0	0·6	0·5	1·03	$1·7\ 10^9$

fication rate that can be attributed to its heterolactic character. The population fell from day 3 of culture.

Lactobacillus plantarum A6 population remained stable for the 6 days of the experiment. The strain produced distinctly more lactic acid than the two other strains, but this was nevertheless limited to 7·4 g l^{-1}. As was previously reported (Giraud *et al.* 1994), *Lactobacillus plantarum* A6 cultured without pH regulation displayed only very slight amylase activity (0·6 U ml^{-1}), resulting in very partial hydrolysis of the starch grains.

Mixed cultures of lactic acid bacteria in a model cassava starch

Mixed cultures of two or three micro-organisms were performed using the same inoculation rate for each strain. The main fermentation parameters are provided in Table 4.

The counting technique and the selective media used enabled independent monitoring of the growth kinetics of each micro-organism in mixed culture (Fig. 3). Whatever the mixture, each micro-organism conserved an identical growth rate to that previously obtained in pure culture. However, maximum population levels decreased. In particular, a decrease in the *Leuc. mesenteroides* population ($2·4\ 10^8$ against $1·7\ 10^9$ bacteria ml^{-1}) during mixed culture of *Lactococcus lactis* and *Leuc. mesenteroides* was noticed (Fig. 3c). Moreover, it was observed that the decline phases of *Leuc. mesenteroides* and *Lactococcus lactis* were accelerated in the presence of *Lactobacillus plantarum* (Fig. 3a, b) compared to the pure cultures. It appears that the interactions between the strains are mainly expressed at the end of fermentation.

It is interesting to note that although *Lactobacillus plantarum* A6 displayed a much smaller growth rate than *Lactococcus lactis* and *Leuc. mesenteroides*, it always attained the same population level and supplanted the two latter micro-organisms from day 2 of fermentation. Indeed, whereas the population of *Lactobacillus plantarum* A6 reached a maximum after 24 h and remained at this level throughout fermentation,

Table 4 Fermentative parameters of mixed cultures

Micro-organism	Min pH	Maximal acidification rate (Δ pH/h)	Lactate g l^{-1}	Acetate g l^{-1}	Ethanol g l^{-1}	μ (h^{-1})	Maximal population cells ml^{-1}
Lactobacillus plantarum A6	3·49	0·32	7·8	0·9	0·5	0·6	$2·4\ 10^9$
Leuconostoc mesenteroides						1·0	$1·2\ 10^9$
Lactobacillus plantarum A6	3·63	0·63	4·6	0·9	—	0·6	$2·5\ 10^9$
Lactococcus lactis						1·1	$1·1\ 10^9$
Lactococcus lactis	3·95	0·56	3·4	0·6	0·3	1·1	$1·3\ 10^9$
Leuconostoc mesenteroides						1·0	$2·4\ 10^8$
Lactobacillus plantarum A6	3·64	0·50	6·0	1·0	0·1	0·6	$2·3\ 10^9$
Lactococcus lactis						1·1	$2·4\ 10^8$
Leuconostoc mesenteroides						1·0	$2·1\ 10^8$

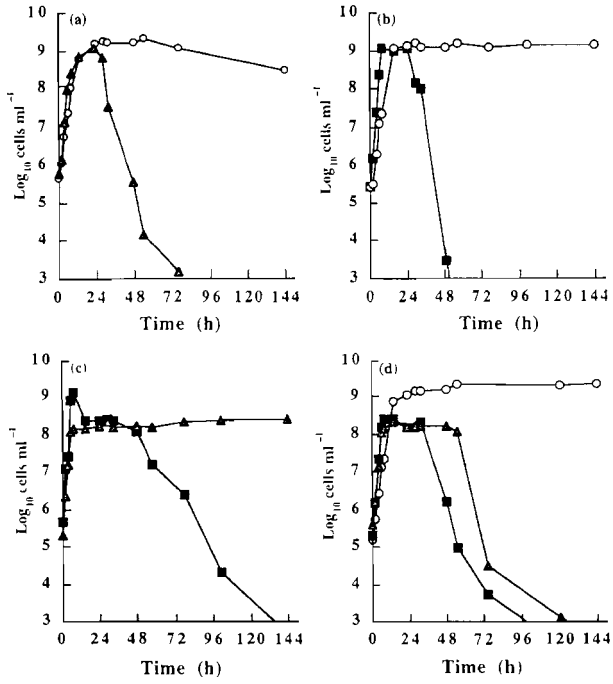

Fig. 3 Specific pattern of growth of each strain during mixed cultures in a model cassava fermentation (a) ○, *Lactobacillus plantarum* A6/▲, *Leuconostoc mesenteroides*, (b) ○, *Lactobacillus plantarum* A6/■, *Lactococcus lactis*, (c) ▲, *Leuc mesenteroides*/■, *Lactococcus lactis*, (d) ○, *Lactobacillus plantarum* A6/▲, *Leuc. mesenteroides*/■, *Lactococcus lactis*

those of *Leuc. mesenteroides and Lactococcus lactis* fell very rapidly from day 2 and were no longer detected after day 3 of fermentation. It is noted that *Leuc. mesenteroides* supplants *Lactococcus lactis* when the two micro-organisms are cultured together.

Rates of acidification of the medium were distinctly higher in mixed culture than in single culture, but the final quantities of lactic acid produced were similar or smaller. In particular, lactate production in the mixed culture of *Lactobacillus plantarum* A6 and *Lactococcus lactis* was less than that in the culture of *Lactobacillus plantarum* A6 alone.

Maximum population decreases of *Lactococcus lactis* and *Leuc. mesenteroides* were observed in the mixed culture of the three strains (d). Nevertheless, their decline phases were slower compared to (a) and (b). *Lactobacillus plantarum* A6 behaved independently with regard to the other micro-organisms present. As before, a succession of different populations was observed during the culture: (i), *Lactococcus lactis* and *Leuc. mesenteroides* were dominant during the first hours of fermentation because of their very rapid growth; (ii) *Lactobacillus plantarum* A6 replaced the two strains from hour 12 onwards, and first *Lactococcus lactis* and then *Leuc. mesenteroides* gradually disappeared.

DISCUSSION

Conventional characterization of lactic acid bacteria during a fermentative process such as retting cassava fermentation (Brauman *et al.* 1996) involves the isolation and then the identification of a considerable number of clones. This is very time-consuming, tedious and sometimes ambiguous Hybridization with DNA probes, as described by Lonvaud-Funel *et al.* (1991) for the specific enumeration of lactic acid bacteria during vinification, seemed to be a good alternative. However, use of these molecular techniques is complex and requires specialized laboratory equipment. Understanding of the phenomena of succession of the different populations involved in these natural fermentation processes is therefore still limited.

The aim of this study was to investigate further the ecology of mixed fermentations and evaluate the effects of mixed starter cultures during cassava fermentation by using model conditions together with a specific counting strategy This selective miniaturized MPN method based on the specific consumption of carbon substrate and resistance to antibiotic enabled us to make a quantitative assessment of the population dynamics of three bacteria species commonly encountered during vegetable fermentation (*Lactobacillus plantarum*, *Leuc. mesenteroides* and *Lactococcus lactis*). This technique offers the advantages of simplicity and suitability for routine purposes However, it remains limited to the use of axenic conditions, the specificity of the media being proposed solely for the three strains initially selected.

This approach showed that whatever the mixed culture, the growth rate of each micro-organism was identical to that in pure culture. In contrast, the acidification rates increased substantially. A succession of microbial populations was also observed; *Lactococcus lactis* and *Leuc. mesenteroides* have high growth rates and dominate the early hours of fermentation before being rapidly replaced by *Lactobacillus plantarum* A6 and disappearing at the end of fermentation. It is interesting to note that this population pattern agrees with that observed during natural cassava fermentation (Brauman *et al.* 1996) and other plant material fermentation such as sauerkraut (Daeschel *et al.* 1987). McDonald *et al.* (1990) consider that the ability of *Lactobacillus plantarum* to maintain a pH gradient between the inside and outside of cells in the presence of large quantities of lactate and acetate would explain why, in most cases, this micro-organism intervenes last in plant fermentation. In contrast, the inability of *Leuc. mesenteroides* to maintain a pH gradient would account for its elimination during the first stage of fermentation.

The early disappearance of *Leuc. mesenteroides* can limit the organoleptic quality of the product during the manufacture of sauerkraut (Harris *et al.* 1992). To overcome this problem, Harris *et al.* (1992) proposed the use of a paired starter culture consisting of a nisin-resistant *Leuc. mesenteroides* strain and a

nisin-producing *Lactococcus lactis* strain to inhibit the growth of *Lactobacillus plantarum* They used a model sauerkraut fermentation to show that sufficient nisin could be produced to reduce the *Lactobacillus plantarum* count to below the level of detection and allow the nisin-resistant *Leuc. mesenteroides* strain to reach maximum cell density.

The strains used in our model were selected, among other reasons, to enable the study in mixed cultures of positive interaction effects such as the breakdown of starch by *Lactobacillus plantarum* A6 and negative interaction effects such as the production of nisin by *Lactococcus lactis* None of these interactions was found during the study. The very low amylase production (0·6 U ml^{-1}) results from the very rapid acidification of the medium which inhibits biosynthesis of the enzyme, as previously reported (Giraud *et al.* 1994) The absence of nisin production can be attributed to several factors such as the rapid acidification of the medium (Yang and Ray 1994), or the carbon source (De Vuyst and Vandamme 1992), or the nitrogen or phosphorus source (De Vuyst and Vandamme 1993) in the medium. The results reported here underline the advantages of using model conditions close to natural fermentation conditions to evaluate the real potential of the micro-organisms selected for use as fermentation starters

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ARTICLE 10

Moulouba F, Lorquin J, Willems A, Hoste B, **Giraud E**, Dreyfus B, Gillis M, de Lajudie P et Boivin C.

Photosynthetic Bradyrhizobia from *Aeschynomene* are specific to stem nodulated species and form a separate 16S rDNA group.

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Photosynthetic Bradyrhizobia from *Aeschynomene* spp. Are Specific to Stem-Nodulated Species and Form a Separate 16S Ribosomal DNA Restriction Fragment Length Polymorphism Group

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We obtained nine bacterial isolates from root or collar nodules of the non-stem-nodulated *Aeschynomene* species *A. elaphroxylon*, *A. uniflora*, or *A. schimperi* and 69 root or stem nodule isolates from the stem-nodulated *Aeschynomene* species *A. afraspera*, *A. ciliata*, *A. indica*, *A. nilotica*, *A. sensitiva*, and *A. tambacoundensis* from various places in Senegal. These isolates, together with 45 previous isolates from various *Aeschynomene* species, were studied for host-specific nodulation within the genus *Aeschynomene*, also revisiting cross-inoculation groups described previously by D. Alazard (Appl. Environ. Microbiol. 50:732–734, 1985). The whole collection of *Aeschynomene* nodule isolates was screened for synthesis of photosynthetic pigments by spectrometry, high-pressure liquid chromatography, and thin-layer chromatography analyses. The presence of *puf* genes in photosynthetic *Aeschynomene* isolates was evidenced both by Southern hybridization with a *Rhodobacter capsulatus* photosynthetic gene probe and by DNA amplification with primers defined from photosynthetic genes. In addition, amplified 16S ribosomal DNA restriction analysis was performed on 45 *Aeschynomene* isolates, including strain BTAi1, and 19 reference strains from *Bradyrhizobium japonicum*, *Bradyrhizobium elkanii*, and other *Bradyrhizobium* sp. strains of uncertain taxonomic positions. The 16S rRNA gene sequence of the photosynthetic strain ORS278 (LMG 12187) was determined and compared to sequences from databases. Our main conclusion is that photosynthetic *Aeschynomene* nodule isolates share the ability to nodulate particular stem-nodulated species and form a separate subbranch on the *Bradyrhizobium* rRNA lineage, distinct from *B. japonicum* and *B. elkanii*.

Rhizobia symbiotically interact with leguminous plants to form nitrogen-fixing nodules most often exclusively occurring on the roots. A few legumes, however, including several *Aeschynomene* species, form nodules also on stem-located sites. The genus *Aeschynomene* includes 22 stem-nodulated species that readily nodulate all along the stem and many other species, considered non-stem nodulated, since their nodulation is restricted to the lower (collar) and submerged part of the stem (6). A number of stem isolates of *Aeschynomene* spp. are of special interest because of their unusual ability to produce photosynthetic pigments, including both bacteriochlorophyll *a* (Bchl *a*) and carotenoids (15, 16, 34, 46). The well-studied strain BTAi1, isolated from stem nodules of *Aeschynomene indica*, was the first bacteriochlorophyll-synthesizing rhizobial strain described (14, 15, 17). When grown aerobically and heterotrophically under a light-dark cycle, strain BTAi1 synthesizes photosynthetic pigments and forms photosynthetic reaction centers like those of the purple nonsulfur photosynthetic bacteria (17, 42). Light-induced CO_2 and light-decreased O_2 uptakes gave evidence of the photosynthetic activity of this strain (17, 26, 27). Because of its functional photosynthetic apparatus, strain BTAi1 can be considered photosynthetic, and by extension, so can all the rhizobia producing photosynthetic pigments.

Rhizobia are taxonomically very diverse. By polyphasic taxonomy, 20 species have been identified and assigned to six genera, *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Bradyrhizobium* and *Azorhizobium* (for a review, see reference 58), and *Allorhizobium* (10). The unusual presence of a photosynthetic system in strain BTAi1 led to the tentative name "*Photorhizobium thompsonum*" (16) or "*Photorhizobium thompsonianum*" (15) for this strain. However, 16S rRNA gene sequence analysis showed that strain BTAi1 was very closely related to both *Bradyrhizobium japonicum* and *Rhodopseudomonas palustris*, suggesting that BTAi1 could be appropriately named *Bradyrhizobium* sp. (*A. indica*) (59). This was later confirmed by additional 16S rRNA gene sequencing of other photosynthetic rhizobia (47, 55) and by fatty acid analysis (47). Additional data came from numerical taxonomy (150 phenotypic characteristics) indicating that the photosynthetic rhizobia constitute a unique phenon that could be considered distinct from *Bradyrhizobium* (33). The precise taxonomic status of *Aeschynomene* photosynthetic rhizobia thus remained unclear.

Each rhizobium can nodulate only a limited number of legumes, referred to as its host range. Depending on the extent of their host range, rhizobia can be considered specific or nonspecific. *Aeschynomene* symbionts comprise both nonspecific rhizobia of the cowpea group and rhizobia specific to the stem-nodulated species (1). No correlation between symbiotic properties and photosynthetic pigment synthesis could be established (48).

Since the different reports on *Aeschynomene* bradyrhizobia generally studied different rhizobium collections and focused on either nodulation (1), phylogeny (55), or photosynthesis (48), the data appeared fragmentary and did not allow a com-

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TABLE 1. Reference strains used in this study[a]

Sp and strain	LMG no	Original host plant	Reference	ARDRA group
Bradyrhizobium japonicum				
NZP5533	6136	*Glycine max*		B
NZP5549^T	6138^T	*Glycine max*		B
USDA135	8321	*Glycine max*		B
Bradyrhizobium elkanii				
NZP5531^T	6134^T	*Glycine max*		C
NZP5532	6135	*Glycine max*		C
Bradyrhizobium sp				
ORS348	12200	*Aeschynomene* sp.	3	D
ORS103	10665	*Faidherbia albida*	13	B
ORS110	10666	*Faidherbia albida*	13	B
ORS121	10677	*Faidherbia albida*	13	C
ORS133	10689	*Faidherbia albida*	13	C
ORS162	10705	*Faidherbia albida*	13	C
ORS169	10712	*Faidherbia albida*	13	B
ORS174	10717	*Faidherbia albida*	13	C
ORS175	10718	*Faidherbia albida*	13	C
ORS180	10719	*Faidherbia albida*	13	Sep.
ORS187	10726	*Faidherbia albida*	13	B
BR29	9520	Unknown		C
BR3621	9966	*Acacia mangium*	37	C
BR4406	9980	*Enterolobium ellipticum*	37	C
INPA9A	10029	*Derris* sp.	37	B

[a] Abbreviations and designations: ORS, I R D Collection, Institut de Recherche pour le Développement, Montpellier, France. LMG, Collection of Bacteria of the Laboratorium voor Microbiologie, University of Ghent, Ghent, Belgium, BR, strain from the CNPBS-EMBRAPA, Centro Nacional de Pesquisa em Biologia do Solo, Seropedica, and Emprasa Brasilhera de Pesquisa Agropequaria, Rio de Janeiro, Brazil. INPA, National Institute of Amazonia Research, Manaus, Brazil, NZP, Culture Collection of the Department for Scientific and Industrial Research, Biochemistry Division, Palmerston North, New Zealand; USDA, U.S Department of Agriculture, Beltsville, Md., Sep , separate

prehensive view of the diversity and evolution of *Bradyrhizobium* isolates from *Aeschynomene* species. Our objective was thus to examine possible links among the presence of photosynthetic pigments, nodulation capacity, and 16S rRNA gene-based phylogeny among bradyrhizobia from *Aeschynomene* species. These three topics were studied with a large collection of isolates. We first enlarged our collection of 45 *Aeschynomene* strains (1–3a) with 78 new bacterial isolates from nodules of diverse native stem- and non-stem-nodulated *Aeschynomene* species from different places in Senegal. We screened the isolates for photosynthetic pigment production (Bchl *a* and carotenoids), for DNA hybridization with a *Rhodobacter capsulatus* photosynthetic gene probe, and for DNA amplification with photosynthetic gene primers. We characterized their host range among *Aeschynomene* species and performed amplified 16S ribosomal DNA (rDNA) restriction analysis (ARDRA) including *B. japonicum*, *Bradyrhizobium elkanii*, and other *Bradyrhizobium* reference strains (1–3, 13, 37). We also determined the 16S rRNA gene sequence of a photosynthetic strain and compared it to sequences of reference strains, including strain BTAi1.

MATERIALS AND METHODS

Bacterial strains and culture growth conditions. The strains used are listed in Tables 1 and 2. By use of the isolation procedure described by Alazard (1), 78 new isolates from *Aeschynomene* species were obtained from naturally occurring root or stem nodules collected in different regions of Senegal. Yeast extract-mannitol medium (54) was the routine medium used for isolation, purification, and maintenance of the rhizobia. Strains were grown at 30°C for 4 to 7 days under aerobic conditions. Type or representative strains of *B. japonicum*, *B. elkanii*, and the various clusters of *Bradyrhizobium* described by Moreira et al. (37) and by Dupuy et al. (13) were included in this study (Table 1).

Nodulation tests. Seeds and plants of *Aeschynomene* species were prepared for root nodulation trials according to previously described procedures (1). Plants were grown under continuous light (20 W/m^2) at 28°C. Four to six plants were tested for each strain. Plants were observed for nodule formation over 6 to 8 weeks, and effectiveness was estimated from visual observation of plant vigor and foliage color.

Photosynthetic pigment determination. Cultures were grown at 30°C for 7 days under aerobic conditions on a 15-h–9-h light-dark cycle. Bchl was extracted under dim light with cold acetone-methanol (7 2 [vol/vol]) at 4°C for 30 min (35). The supernatant was analyzed with a Beckman DU40 spectrophotometer. Absorption spectra were generated by scanning over a wavelength range from 350 to 800 nm. Carotenoids were further purified and analyzed by high-pressure liquid chromatography (HPLC) or thin-layer chromatography (TLC) as previously described (35).

Southern hybridization. Genomic DNA was extracted as previously described (36). Total DNA was digested by *Eco*RI, *Pst*I, or *Hin*dIII as specified by the manufacturer (Boehringer Mannheim or Pharmacia). Restricted DNA was run in a 0.8% agarose gel and transferred to a nylon membrane under alkaline conditions by the Southern blot standard procedure (43). Hybridization was carried out with the digoxigenin labeling and detection kit from Boehringer Mannheim. The probe was labeled by randomly primed incorporation of digoxigenin-linked dUTP, (DIG-dUTP) and hybridization was performed overnight at 37°C in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 50% (vol/vol) deionized formamide, 2% (wt/vol) blocking reagent in maleic acid buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5), 0.1% (wt/vol) *N*-lauroylsarcosine (Sarkosyl), and 0.02% (wt/vol) sodium dodecyl sulfate. After stringency washes, hybrids were revealed by a chemiluminescence reaction, and detection was performed on X-ray film. The hybridization probe was a 3.3-kb *Eco*RI-*Hin*dIII fragment (*pufBALMX*) from the plasmid pUC13::*pufBALMX* (5, 8) provided by A. Lilburn (University of British Columbia, Vancouver, Canada). This fragment is part of the 46-kb region of the *R. capsulatus* chromosome which encodes the photosynthetic apparatus.

PCR amplification of *puf* genes. Part of the *pufLM* genes was amplified from genomic DNA with the nondegenerated primers pufL278f (5′-CACCCATCTC GATTGGGTGTCG-3′) and pufM278r (5′-CTCCAGCTGCCCATGAAGATC G-3′), specifically defined from the *pufLM* sequence of *Bradyrhizobium* sp. strain ORS278 and amplifying a 926-bp fragment from the 3′ end of *pufL* and the 5′ end of *pufM* in the ORS278 sequence (20a). Amplification reactions were performed in a 50-μl final volume containing 0.1 μg of DNA, each deoxyribonucleoside triphosphate at a concentration of 0.2 mM, 0.8 μM (each) primer, 1.5 mM $MgCl_2$, 1.25 U of *Taq* DNA polymerase (Gibco BRL), and the buffer supplied

TABLE 2 Nodulation specificity, Bchl *a* and carotenoid (Crt) content, and ARDRA grouping of bradyrhizobia from *Aeschynomene*

Cross-inoculation group and host plant	Bacterial strain[a]	LMG no.	Reference or source	Nodulation on group[d]				Crt type spectrum[e]	ARDRA group[f]
				I (*A. elaphroxylon*)	II (*A. afraspera*)	III			
						A. indica	*A. sensitiva*		
Group I									
A. americana	ORS301 (R)[b]	8290	1	E	e	0	0	W	B
A elaphroxylon	ORS377 (C)	15420	This study	E	i	0	0	W	
	ORS378 (C)	15421	This study	E	ı	0	0	W	
	ORS379 (C)	15422	This study	E	i	0	0	W	
	ORS381 (C)	15423	This study	E	i	0	0	W	
	ORS304 (C)[c]	8069	1	E	e	i	0	W	Sep.
A pfundii	ORS302 (C)[b]	10296	1	E	E	0	0	W	
A schimperi	ORS271 (R)		This study	nt	E	0	0	W	
	ORS272 (R)	15408	This study	nt	E	0	0	W	
	ORS275 (R)	15410	This study	nt	E	0	0	W	
	ORS273 (R)		This study	nt	e	0	0	W	
	ORS274 (R)	15409	This study	nt	e	0	0	W	
	ORS305 (R)[b]	8291	2	E	e	0	0	W	Sep.
	ORS343 (R)[b]	11801	3	nt	e	0	0	W	
	ORS360 (R)		D. Alazard	nt	0	0	0	W	
A. uniflora	ORS309 (C)	7838, 8070	2	E	e	0	0	W	C
	ORS355 (C)[b]	10301	3	nt	e	0	0	W	
Group II									
A. ciliata	ORS332 (U)	8296	D. Alazard	0	e	0	0	nt	
	ORS333 (U)	8307, 10289	D. Alazard	0	e	ı	i	nt	
A nilotica	ORS358 (U)	10303	D. Alazard	nt	e	0	0	W	C
	ORS364 (S)	11802	3	nt	e	E	i	LP	
A afraspera	ORS313 (R)	15416	D. Alazard	i	e	0	0	W	
	ORS336 (S)[b]	8298, 11800	3	ı	e	0	0	W	C
	ORS284 (S)		This study	nt	e	0	0	W	
	ORS317 (R)	15417	D Alazard	nt	e	0	0	W	
	ORS349 (U)	10299	D. Alazard	nt	e	0	0	W	
	ORS354 (S)	15424	D. Alazard	nt	e	0	0	W	
	ORS347 (S)	10298	3	nt	E	0	0	W	
	ORS323 (S)	15380	D. Alazard	nt	i	0	0	LP	
	ORS325 (S)	8304	D. Alazard	nt	i	e	E	LP	
	ORS288 (S)	12189, 15412	This study	nt	e	e	i	LP	
	ORS289 (S)	15413	This study	nt	e	e	e	LP	
	ORS290 (S)	15414	This study	nt	e	e	e	LP	
	ORS363 (S)	15418	This study	nt	e	e	e	W	
	ORS365 (S)	15419	This study	nt	e	e	e	LP	
	ORS291 (S)	15415	This study	nt	e	e	E	LP	
	ORS308 (R)[c]	8293, 15401	2	nt	E	e	E	LP	A
	ORS303 (S)	8292	1	0	E	e	ı	LP	
	ORS322 (S)[c]	8073, 15403	1	0	E	e	i	LP	A
	ORS312 (R)[c]	8294	2	nt	E	e	i	LP	A
	ORS324 (R)[c]	8295	3	nt	E	e	i	LP	A
	ORS337 (R)[c]	8299 t2	2	nt	E	e	i	LP	A
	ORS374 (S)	11960	This study	nt	E	E	i	O	
	ORS286 (S)	15377	This study	nt	E	E	e	LP	
	ORS351 (S)	10300	3	nt	E	E	e	LP	
	ORS285 (S)	15376	This study	0	E	E	E	LP	
	ORS287 (S)	15378 t1	This study	nt	E	E	E	LP	A
	ORS335 (U)	8297	D. Alazard	nt	E	E	E	LP	
	ORS352 (S)	15384	D. Alazard	nt	E	E	E	LP	
	ORS353 (S)	15385	D. Alazard	nt	E	E	E	LP	
	ORS357 (U)	10302	D. Alazard	nt	E	E	E	LP	
	ORS362 (S)	10305	3	nt	E	E	E	LP	
	ORS356 (S)	15386	This study	nt	e	E	E	LP	
Group III									
A tambacoundensis	**ORS266** (S)	15442	This study	0	0	e	e	LP	A
	ORS268 (S)		This study	0	e	e	E	LP	
	ORS331 (S)[c]	11799	3	0	0	E	E	LP	A
	ORS334 (S)[c]	8308	1	0	0	E	E	LP	
A indica	**ORS280** (S)	15411	This study	0	0	e	e	LP	
	ORS306 (S)[c]	8300, 11797, 10286	1	0	0	e	e	LP	A
	ORS307 (S)	15379	This study	0	0	e	e	LP	
	ORS310 (S)[c]	8071 t1	1	0	0	e	e	LP	
	ORS311 (S)		This study	0	0	e	e	LP	
	ORS318 (S)[c]	8301 t1	2	0	0	e	e	LP	
	ORS319 (S)[c]	8302 t1	2	0	0	e	e	LP	
	ORS339 (S)		This study	0	0	e	e	LP	
	ORS375 (S)	15388	This study	0	0	e	e	LP	
	ORS397 (S)		This study	0	0	e	e	LP	

Continued on following page

TABLE 2—*Continued*

Cross-inoculation group and host plant	Bacterial strain[a]	LMG no	Reference or source	Nodulation on group and sp[d]				Crt type spectrum[e]	ARDRA group[f]
				I (*A elaphroxylon*)	II (*A afraspera*)	III			
						A indica	*A sensitiva*		
	ORS320 (S)[c]	8303	2	0	E	e	e	LP	A
	ORS321 (S)		This study	0	0	e	e	LP	
	ORS328 (S)[c]		1	0	0	e	E	LP	
	ORS338 (S)		3	0	0	e	E	LP	
	ORS376 (S)		This study	0	0	e	E	LP	
	ORS383 (S)		This study	0	0	e	E	LP	
	ORS386 (S)		This study	0	0	e	E	LP	A
	ORS400 (S)		This study	0	0	E	E	LP	
	BTAi1 (S)		14	0	0	E	e	LP	A
	ORS340 (S)		This study	0	0	E	e	LP	
	ORS341 (S)		This study	0	0	E	e	LP	
	ORS372 (S)	11805	This study	0	0	E	e	LP	
	ORS388 (S)	11814	This study	0	0	E	e	LP	
	ORS389 (S)	15393	This study	0	0	E	e	LP	
	ORS390 (S)	11813	This study	0	0	E	e	LP	A
	ORS391 (S)	15394	This study	0	0	E	e	LP	
	ORS392 (S)	11812, 12205	This study	0	0	E	e	O	A
	ORS393 (S)	11811, 15407	This study	0	0	E	e	LP	A
	ORS394 (S)	15395	This study	0	0	E	e	LP	
	ORS269 (S)		This study	0	0	E	E	LP	
	ORS270 (S)		This study	0	0	E	E	LP	
	ORS282 (S)		This study	0	0	E	E	O	
	ORS342 (S)	15383	This study	0	0	E	E	LP	
	ORS344 (S)	12198	This study	0	0	E	E	DP	
	ORS346 (S)	12199	This study	0	0	E	E	LP	
	ORS371 (S)	11804, 12203	This study	0	0	E	E	DP	A
	ORS373 (S)	11806	This study	0	0	E	E	LP	
	ORS382 (S)	11816, 15406	This study	0	0	E	E	LP	A
	ORS384 (S)	15390	This study	0	0	E	E	LP	A
	ORS385 (S)	15391	This study	0	0	E	E	LP	
	ORS387 (S)	15392	This study	0	0	E	E	LP	
	ORS395 (S)	15396	This study	0	0	E	E	LP	
	ORS396 (S)	11808	This study	0	0	E	E	LP	
	ORS399 (S)	12206	This study	0	0	E	E	LP	
	ORS327 (S)	15381	This study	0	0	ı	e	LP	
	ORS368 (S)	15387	This study	0	0	e	ı	LP	A
	ORS398 (S)	11809	This study	0	ı	E	ı	O	
	ORS380 (S)	11817	This study	0	E	E	ı	LP	
A. sensitiva	**ORS297** (S)	12195	This study	0	0	e	e	LP	A
	ORS279 (S)	12188	This study	0	0	e	E	O	A
	ORS292 (S)	12205	This study	0	0	e	E	LP	
	ORS294 (S)	12192	This study	0	0	e	E	LP	A
	ORS330 (S)[c]	8306, 15404	1	0	0	e	E	LP	
	ORS293 (S)	12191	This study	0	0	E	e	O	
	ORS300 (S)	12197, 15400	This study	0	E	E	e	LP	A
	ORS295 (S)		This study	0	0	E	e	LP	
	ORS296 (S)	12194	This study	0	0	E	e	DP	D
	ORS298 (S)	12196	This study	0	0	E	e	LP	A
	ORS299 (S)	15399	This study	0	0	E	e	LP	Sep.
	ORS278 (S)	12187	This study	0	0	E	E	O	A
	ORS276 (S)	12185	This study	0	0	E	E	LP	
	ORS277 (S)	12186	This study	0	0	E	E	O	A
	ORS359 (S)	12201	This study	0	0	E	E	LP	A
	ORS361 (S)	12202	This study	0	0	E	E	LP	A

[a] Photosynthetic strains are in boldface. R, strain isolated from root nodules; C, strain from nodule located on the stem collar (nodule on the lower and submerged part of the stem); S, strain from stem nodule; U, unknown. ORS, I.R.D. Collection, Institut de Recherche pour le Développement, Montpellier, France; LMG, Collection of Bacteria of the Laboratorium voor Microbiologie, University of Ghent, Ghent, Belgium.

[b] Non-free-living nitrogen-fixing strain (2, 3).

[c] Free-living nitrogen-fixing strain (2, 3).

[d] nt, not tested; 0, no nodulation; ı, ineffective root nodulation; e, partially effective root nodulation; E, effective root nodulation.

[e] Crt, carotenoid. The spectrum is described in Fig. 1. W, white (strain lacking Bchl *a* and carotenoid); DP, dark pink; O, orange; LP, light pink.

[f] Sep., separate.

with the enzyme. The amplification conditions were 5 min at 94°C, 30 cycles consisting of 30 s at 94°C and 30 s at 60°C, and 1 min at 72°C.

ARDRA. DNA was extracted according to the procedure of Pitcher et al. (41) with slight modifications. A loopful of cells was washed with 500 μl of 150 mM NaCl–10 mM EDTA, pH 8.0. DNA was extracted by a procedure involving lysis with Sarkosyl-guanidinium thiocyanate (Sigma), phenol-isoamylic alcohol treatment, and isopropanol precipitation. DNA was further purified by treatment for 1 h at 37°C with RNase at a final concentration of 250 μg/ml. Milli-Q water (Millipore) was used for all enzymatic reactions and amplification procedures.

ARDRA was performed as described by Vaneechoutte et al. (49). 16S rDNA was amplified with a forward primer (5′-AGAGTTTGATCATGGCTCAG-3′) and a reverse primer (5′-TACCTTGTTACGACTTCACCCCA-3′) supplied by Pharmacia. PCR was carried out in a 50-μl reaction volume by mixing 250 ng of template DNA with the polymerase reaction buffer (10 mM Tris-HCl, 1.5 mM

$MgCl_2$ 50 mM KCl, pH 8.4), 10 nmol of each deoxynucleoside triphosphate (Pharmacia), 20 pmol of each primer (Pharmacia), and 1.25 U of Euro*Taq* polymerase (Eurogentec, Seraing Belgium). Amplification was achieved in a Perkin-Elmer PCR GeneAmp 9600 thermocycler (Perkin-Elmer Cetus) with the following temperature profile: an initial denaturation at 95°C for 7 min, 50 cycles of denaturation (45 s at 95°C), annealing (30 s at 57°C), and extension (2 min at 72°C), and a final extension at 72°C for 10 min. The PCR products were purified with the PCR Clean Up kit (Boehringer) according to the manufacturer's recommendations.

Restriction was carried out as specified by the manufacturers in 20-μl volumes of commercially supplied incubation buffer containing 10 μl of PCR product and 5 U each of restriction endonucleases *Hin*fI, *Dde*I, and *Mwo*I (New England Biolabs, Leusden, The Netherlands) or *Alu*I and *Hha*I (Pharmacia). Restriction fragment length polymorphism patterns were analyzed by horizontal gel electrophoresis of each restriction mixture at 90 V for 130 min in 2% (wt/vol) Metaphor agarose (FMC Bioproducts, Rockland, Maine) in TBE buffer (Tris-HCl, 89 mM, boric acid, 89 mM, EDTA, 2 mM, pH 8.0) containing 0.5 μg of ethidium bromide per ml. Gels were viewed under UV illumination (312 nm) and photographed with a charge-coupled device camera (768 by 494 pixels). Images were scanned for normalization of restriction patterns with the Gelcompar 3.1 software package (53) with *Alu*I-digested pBR322 as molecular weight markers. For each strain, the five normalized restriction patterns were assembled into a combined profile and analyzed with the Dice similarity coefficient (S_D) expressed as a percentage and the unweighted pair group method with average linkage (UPGMA) clustering algorithm.

Analysis of the 16S rRNA genes. A few colonies of strain ORS278 (LMG 12187) were suspended in 50 μl of water, and a small amount of sterile glass beads was added. The suspension was mixed for 1 min, boiled for 5 min, and again mixed for 1 min, and finally, the cell debris was spun down. Five microliters of the supernatant was used in a PCR to amplify the nearly complete 16S rRNA gene (positions 28 to 1521 of the *Escherichia coli* 16S rRNA gene). The PCR product was purified with a Prep-A-Gene kit (Bio-Rad Laboratories, Hercules, Calif.) and sequenced with primers to universally conserved fragments, a *Taq* dye-deoxy terminator cycle sequencing kit (Perkin-Elmer Corp., Foster City, Calif.), and an automatic DNA sequencer (model 377, Perkin-Elmer Corp.). The obtained sequence fragments were aligned, and a consensus sequence was constructed with the program AutoAssembler (Perkin-Elmer). For further phylogenetic analysis, the Genetics Computer Group (GCG) package (12) and the phylogeny inference (PHYLIP) package (18), available on the Belgian EMBnet Node of the Brussels Free University Computing Centre, were used. The new sequence was aligned, together with reference sequences obtained from the EMBL data library, with the program PILEUP of the GCG package. In total, a continuous stretch of 1,401 base positions (including gaps) was used for further analysis. Distances, modified according to the Kimura-2 model, were calculated by using the DNADIST program of the PHYLIP package, and the program NEIGHBOR of the same package was used to produce an unrooted phylogenetic tree. The stability of the groupings was verified by bootstrap analysis (500 replications) with the PHYLIP programs DNABOOT, DNADIST, NEIGHBOR, and CONSENSE.

Nucleotide sequence accession number. The EMBL accession number for the 16S rRNA gene sequence of strain ORS278 (LMG 12187) is AJ133779.

RESULTS

Isolation of rhizobia from *Aeschynomene* spp. We obtained nine isolates from root or collar nodules of non-stem-nodulated *Aeschynomene* species (*A. elaphroxylon*, *A. uniflora*, or *A. schimperi*) and 69 root or stem nodule isolates from the stem-nodulated *A. afraspera*, *A. ciliata*, *A. indica*, *A. nilotica*, *A. sensitiva*, and *A. tambacoundensis* (Table 2) from various places in Senegal. Included in this study were 45 isolates from various *Aeschynomene* species previously isolated by Alazard (1–3a). Growth on yeast-mannitol agar produced small typical *Bradyrhizobium*-like colonies after incubation at 30°C for 5 to 7 days. Colonies formed by numerous isolates turned either light pink (LP), dark pink (DP), or orange (O; see below and Table 2), especially after light exposure, suggesting photosynthetic pigments (15, 35).

Host-specific nodulation within the genus *Aeschynomene*. On the basis of nodulation tests performed with 15 rhizobial strains and 20 different *Aeschynomene* species, Alazard (1) identified three cross-inoculation groups among *Aeschynomene* species. Group I contained only non-stem-nodulated *Aeschynomene* species, while true stem-nodulated *Aeschynomene* spp. belonged to groups II and III. Strains isolated from *Aeschynomene* spp. of groups I and II were able to nodulate plants from other cross-inoculation groups, whereas rhizobia isolated from group III plants nodulated only plants of the homologous cross-inoculation group.

To confirm and extend these results, all the new *Aeschynomene* isolates (except ORS326 and ORS348) were tested for root nodulation on plants representative of nodulation group I (*A. elaphroxylon*), group II (*A. afraspera*), and group III (*A. indica* and *A. sensitiva*) (Table 2).

Isolates from *A. americana*, *A. elaphroxylon*, *A. pfundii*, *A. schimperi*, and *A. uniflora* nodulated *A. elaphroxylon* (group I) and *A. afraspera* (group II) but rarely plants of group III. According to the work of Alazard (1), we thus classified *A. schimperi* and *A. uniflora* in cross-inoculation group I together with *A. americana*, *A. elaphroxylon*, and *A. pfundii*. Isolates from *A. ciliata* nodulated *A. afraspera* (group II) and generally representative plants of group III (*A. indica* and *A. sensitiva*). *A. ciliata*, previously classified in cross-inoculation group III by Alazard (1), thus rather belongs to group II, comprising *A. nilotica* and *A. afraspera*. Isolates from plants of group III (*A. tambacoundensis*, *A. indica*, and *A. sensitiva*) all nodulated group III representatives but never nodulated *A. elaphroxylon* from group I and hardly ever nodulated *A. afraspera* from group II. Their nodulation ability thus appeared to be restricted to group III plants.

Bchl *a* synthesis is a specific characteristic of group III stem-nodulated *Aeschynomene* symbionts. To evaluate the extent of photosynthesis among *Aeschynomene* isolates and to determine whether there is a relationship between the photosynthetic nature of the microsymbiont and the host plant or the host plant cross-inoculation group, we screened our collection for Bchl *a* and carotenoid content by spectrometry, HPLC, and TLC analyses.

Absorption spectra from *Aeschynomene* isolates were obtained by using acetone-methanol extracts of bacterial cells. All strains originating from *Aeschynomene* species belonging to group III (*A. tambacoundensis*, *A. indica*, and *A. sensitiva*) produced an absorbance peak at 770 nm, characteristic of Bchl *a*, and peaks around 400 to 500 nm, corresponding to carotenoids. These peaks were absent in all strains originating from the group I plants. The content of isolates originating from group II plants appeared variable: 70% of the strains synthesized Bchl *a* and carotenoids. Three different absorption spectra, corresponding to three pigmentation groups, were obtained for Bchl-synthesizing strains. LP, DP, and O strains (Table 2) exhibited spectra A, B, and C, respectively (Fig. 1). The determination of the carotenoid composition of several representative strains of each group by TLC and HPLC analysis confirmed our previous results (35). R_f (TLC) and retention time (HPLC) values determined for each pigment were found to be identical to those already found. Bchl *a* and carotenoid contents were also determined by spectrophotometry and HPLC analysis and were found to have values similar to those of our previous report (35). LP strains produced only spirilloxanthin, whereas DP and O strains synthesized both spirilloxanthin and canthaxanthin, together with several other minor carotenoids. The difference in pigmentation between DP and O strains is due to the different ratios of canthaxanthin to spirilloxanthin in these strains. This ratio was found to be 88 to 93% for O strains and 70 to 77% for DP strains, thus confirming our previous observations (35).

Up to now, Bchl *a* has been found in only photosynthetic organisms (20, 24, 39, 40, 45). Consequently, Bchl-containing *Aeschynomene* strains will be referred to as photosynthetic strains in the following sections.

Among isolates originating from group II plants, the photosynthetic rhizobia are those nodulating *A. indica* and *A. sensi-*

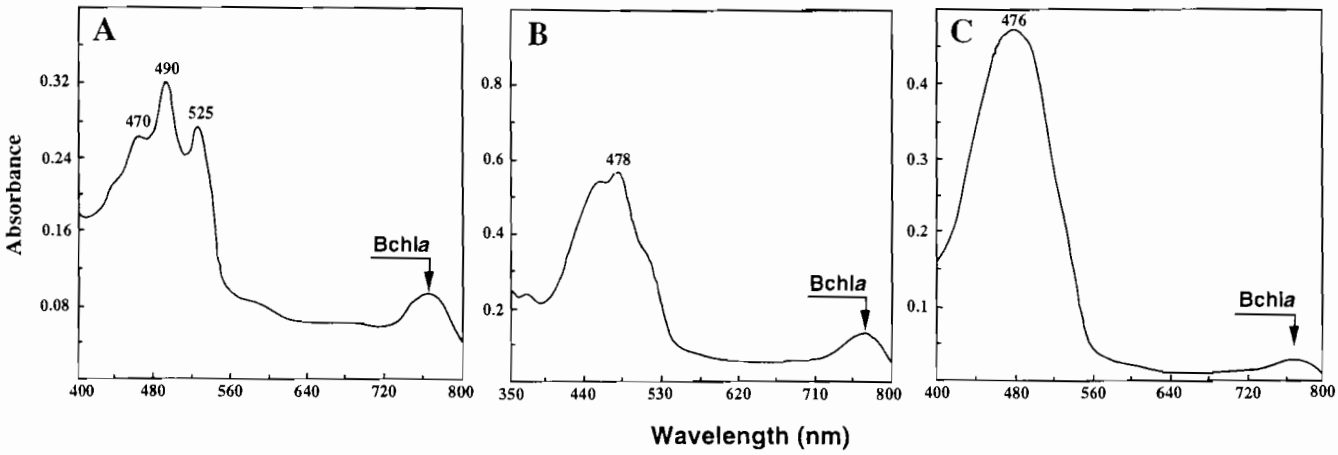

FIG 1 Absorption spectra of acetone-methanol extracts from stem-nodulating photosynthetic rhizobia (A) Extract from LP strain ORS266; (B) extract from DP strain ORS397, (C) extract from O strain ORS277 Each extract was obtained from a 50-ml culture.

tiva, representatives of group III plants (with two exceptions being ORS323 and ORS333). A high correlation was thus found between the ability to synthesize Bchl *a* and the ability to nodulate *A. indica* and *A. sensitiva*, suggesting a relationship between the rhizobial photosynthetic nature and nodulation ability. It should also be noticed that the Bchl *a*-synthesizing strains from *A. afraspera* were more effective than the nonphotosynthetic strains.

Genetic evidence for the presence of bacterial photosynthetic genes in *Aeschynomene* stem-nodulating bradyrhizobia. The photosynthetic apparatus of purple nonsulfur bacteria (belonging to the alpha subclass of the *Proteobacteria* together with *Bradyrhizobium*) is mainly composed of pigment-protein complexes, namely, reaction center and light-harvesting complexes (see reference 50 for a review). To evaluate the occurrence of genes encoding photosynthetic proteins in Bchl-synthesizing rhizobia, we screened 16 selected *Aeschynomene* strains for the presence of DNA sequences hybridizing to probes consisting of the *pufBALMX* genes from *R. capsulatus* (5). The *pufBALMX* genes have been shown to encode the α- and β-polypeptides of the light-harvesting complex B875 (genes *pufBA*), the reaction center polypeptides (genes *pufLM*), and an open reading frame (*pufX*) (4, 5, 60). Genomic DNA from photosynthetic *Aeschynomene* strains ORS266, ORS277, ORS278, ORS294, ORS306, ORS322, ORS364, ORS371, and BTAi1 hybridized with the *pufBALMX* probe. Conversely, genomic DNA from nonphotosynthetic strains ORS301, ORS304, ORS305, ORS309, ORS347, ORS358, and ORS377 did not show any detectable hybridization with this probe (results not shown).

The presence of *puf* genes among *Aeschynomene* isolates was also evaluated by *pufLM* partial amplification with primers defined from the *pufLM* sequence of *Bradyrhizobium* sp. strain ORS278. All the photosynthetic strains studied (ORS266, ORS268, ORS277, ORS278, ORS282, ORS285, ORS287, ORS294, ORS296, ORS300, ORS306, ORS320, ORS322, ORS324, ORS330, ORS335, ORS344, ORS352, ORS353, ORS357, ORS362, ORS363, ORS364, ORS368, ORS371, and ORS380) gave a fragment of the expected 926-bp size, while the nonphotosynthetic strains studied (ORS292, ORS301, ORS302, ORS304, ORS305, ORS309, ORS336, ORS347, and ORS358) gave no amplification band.

ARDRA. Nearly full-length 16S rDNAs from 46 *Aeschynomene* nodule isolates (including BTAi1) and from 19 reference strains of *B. japonicum*, *B. elkanii*, and other *Bradyrhizobium* spp. previously characterized (13, 37) were amplified, yielding an expected single band of about 1,500 bp (data not shown). The amplified 16S rDNA of all strains was restricted with the enzymes *Hin*fI, *Dde*I, *Mwo*I, *Alu*I, and *Hha*I. The combined restriction patterns were used to construct a dendrogram based on the UPGMA algorithm (Fig. 2).

At or above a mean Dice similarity coefficient (S_D) value of ± 88%, four main clusters were delineated. Except for ORS296 and ORS299, all photosynthetic *Aeschynomene* strains belong to the large cluster A. *B. japonicum* constituted cluster B together with two *Aeschynomene* strains, ORS301 (nonphotosynthetic) and ORS326 (photosynthetic status not determined); one strain isolated from *Derris* sp. (LMG 10029); and four strains from *Faidherbia albida* (ORS103, ORS110, ORS169, and ORS187). Cluster C contained *B. elkanii*, three nonphotosynthetic strains of *Aeschynomene* spp. (ORS309, ORS336, and ORS358), strain LMG 9520, and strains isolated from diverse other hosts including *Enterolobium ellipticum* (LMG 9980), *Acacia mangium* (LMG 9966), and *F. albida* (ORS121, ORS133, ORS162, ORS174, and ORS175). The strains ORS296 (photosynthetic) and ORS348 (photosynthetic status not determined) formed cluster D, and the photosynthetic strain ORS299 is the closest relative of this cluster. No evident relationship between the original host plant and the ARDRA clustering could be found.

16S rRNA gene sequence analysis. Strain ORS278 (LMG 12187) was chosen as a representative of the photosynthetic strains, and its 16S rRNA gene sequence was determined. It consisted of 1,441 nucleotides and was very similar to the sequence of the photosynthetic strains BTAi1 (11 differences) and USDA 4377 (5 differences). A phylogenetic tree was constructed to determine the position of this strain among other bradyrhizobia (Fig. 3). Strain ORS278 (LMG 12187) formed a separate cluster together with *Bradyrhizobium* strains BTAi1 and USDA 4377 and *Blastobacter denitrificans* LMG 8443. This grouping was supported by a bootstrap value of 100% and was distinct from *B. japonicum* and *B. elkanii*.

DISCUSSION

Since the isolation of the strain BTAi1, which displays heterotrophic photosynthesis (15, 17, 28), several bradyrhizobia from various *Aeschynomene* species have been reported to be photosynthetic, which is a rare property among rhizobia (for a

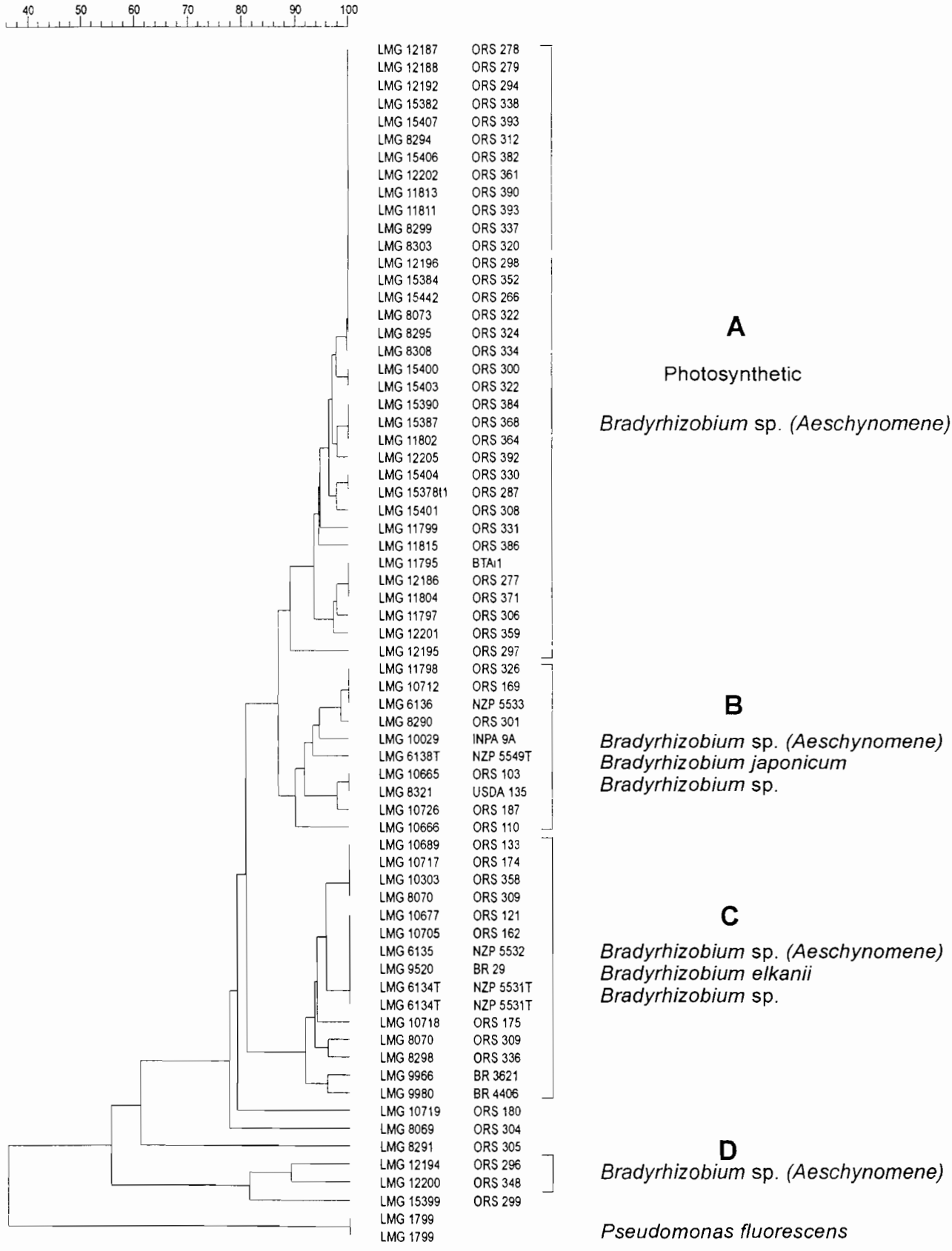

FIG. 2 ARDRA results presented as a dendrogram based on S_D values, calculated by UPGMA. *Pseudomonas fluorescens* LMG 1799, present in our database, was included as an outgroup organism

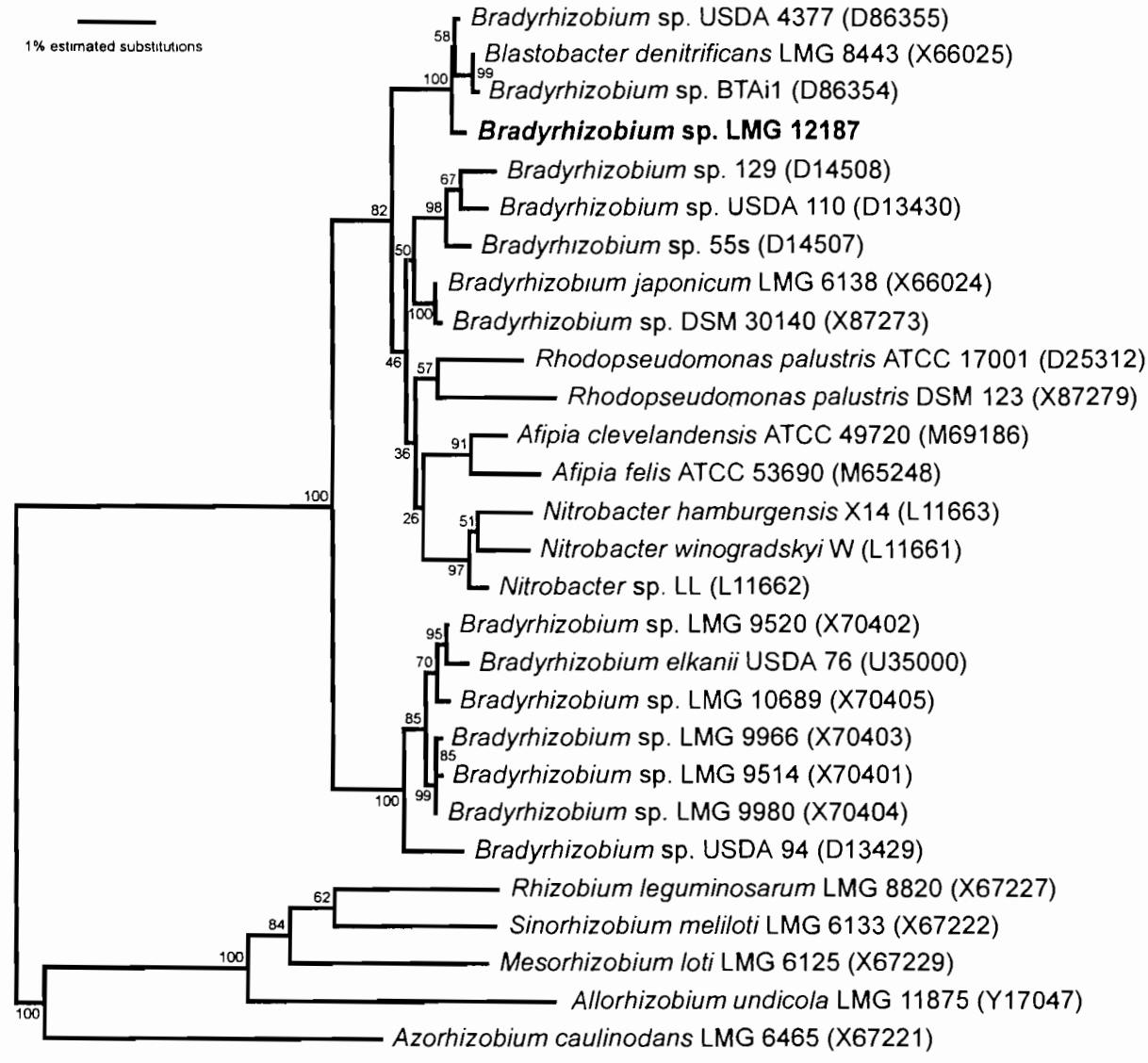

FIG. 3 Neighbor-joining dendrogram showing the position of photosynthetic strain ORS278 (LMG 12187) among bradyrhizobia and closely related taxa Bootstrap values, expressed as percentages of 500 replications, are given at the branching points Numbers in parentheses are the accession numbers of the sequences used. The bar represents one estimated substitution per 100 nucleotide positions

review, see reference 19). Phylogenetic investigations established that the photosynthetic rhizobia belonged to the *B. japonicum-R. palustris* lineage (55). Previously, nodulation investigations showed that a group of *Aeschynomene* bradyrhizobia specifically nodulated stem-nodulated species (1). However, no correlation among photosynthetic ability, phylogenetic position, and host specificity could be established, mainly because the different results were established with different bradyrhizobial collections.

In this study, by characterizing a collection of isolates from the genus *Aeschynomene*, specifically by determining their Bchl content, nodulation abilities, and 16S rRNA gene-based phylogeny, we demonstrate that photosynthetic rhizobia are mainly monophyletic and share the ability to nodulate particular stem-nodulated *Aeschynomene* species.

To obtain more photosynthetic isolates, we extended the Senegalese collection of *Aeschynomene* rhizobia (1–3a), mainly by isolating bacteria from naturally occurring stem nodules, since photosynthetic rhizobia are generally isolated from stem-nodulated *Aeschynomene* spp. When grown under a light-dark cycle, nearly all the stem isolates examined (Table 2) were found to produce Bchl *a*, a photosynthetic pigment found in only photosynthetic organisms (20, 24, 39, 40, 45), confirming previous reports suggesting that photosynthesis is widespread among stem-nodulating strains (34). The photosynthetic nature of the Bchl-synthesizing bradyrhizobia was confirmed by both Southern hybridization and gene amplification studies. Indeed, the presence of DNA sequences homologous to reaction center and light-harvesting genes from *R. capsulatus* was detected in all Bchl-synthesizing strains examined, while the presence of *pufLM* genes in Bchl-synthesizing strains was evidenced by DNA amplification with *pufLM* primers designed from the *pufLM* sequence of *Bradyrhizobium* sp. strain ORS278 (20a). Although the primers used were not designed from a conserved motif in the *puf* genes, they were found suitable to amplify a *puf* fragment from all the photosynthetic bradyrhizobia tested in this study. All Bchl-synthesizing strains produced the carotenoid spirilloxanthin, which is known to be

bound to the light-harvesting protein-associated complex in purple nonsulfur bacteria and members of the family *Chromatiaceae* (9, 21–23). A few of them also synthesized other carotenoids, including canthaxanthin (35). The role of the carotenoid canthaxanthin in photosynthesis is unknown, but this pigment has great biotechnological value (38).

Within the past 15 years, the taxonomy of the rhizobia has greatly changed with the discovery of several new species and genera (58). Quite a number of diverse nodule isolates have been characterized and described in the literature as belonging to the large group of bradyrhizobia (13, 37, 52), but only a few studies brought sufficient taxonomic data for clear taxonomic conclusions and nomenclatural decisions (30–32, 56). Several authors have reported the difficulties encountered in studying bradyrhizobia and contradictory results from phenotypic and genotypic studies (13, 33). Here we add further taxonomic data on a collection of 123 isolates from *Aeschynomene* species, either stem nodulated or non-stem nodulated, together with 19 *Bradyrhizobium* reference strains, including *B. japonicum* (30), *B. elkanii* (32), and *Bradyrhizobium* sp. strains partially characterized in the literature (13, 37, 52).

Alazard (3) showed that the free-living nitrogen-fixing *Aeschynomene* symbionts form a single phenon within the *Bradyrhizobium* genus. Moreover, two representative strains of this phenon, ORS310 and ORS322, were able to grow in the free-living state at the expense of N_2 (2), like *Azorhizobium caulinodans*, which is highly specialized in the stem nodulation of *Sesbania rostrata* (6). Our results demonstrate that these two free-living nitrogen-fixing *Aeschynomene* bradyrhizobia also synthesize Bchl *a* (Table 2) These observations corroborate the work of Ladha and So (33), who found that 52 photosynthetic *Aeschynomene* nodule isolates belonging to a separate phenon had the ability to grow and fix N_2 in the absence of combined nitrogen. Therefore, diazotrophy is probably a general property of photosynthetic isolates, both properties together probably conferring a great selective saprophytic advantage on these bacteria.

We observed a strong correlation between photosynthetic and nodulation abilities. Indeed, all the photosynthetic strains were isolated from stem-nodulated *Aeschynomene* species belonging to cross-inoculation groups II and III (1, 6). Moreover, among isolates originating from stem-nodulated *Aeschynomene* spp. of group II, the photosynthetic strains corresponded to those which are also able to nodulate plants of group III (*A. sensitiva* and *A. indica*). In contrast to the photosynthetic strains, the nonphotosynthetic rhizobia isolated from plants of groups I and II were able to nodulate *F. albida* and thus belong to the cowpea group (data not shown). This study thus confirms the occurrence of nonspecific and specific bradyrhizobia among *Aeschynomene* symbionts with the photosynthetic strains being highly specific. In rhizobium-legume interactions, host specificity is mainly controlled by extracellular bacterial signal molecules, which are called Nod factors (see references 11 and 51 for reviews). All *Bradyrhizobium* Nod factors examined so far bear a substituted or a nonsubstituted methyl fucose group on their reducing ends (7, 18a, 44). Specific *Aeschynomene* photosynthetic symbionts thus represent an interesting model to determine which structural features of Nod factors account for host specificity.

From our 16S rDNA-based phylogenetic analysis (Fig. 3), it is apparent that the photosynthetic strains, represented by strains ORS278 (LMG 12187), BTAi1, and USDA 4377, form a separate cluster together with *B. denitrificans*, an unpigmented budding organism from lake water (25). This small group is supported by a bootstrap value of 100%. In a separate analysis, in which a shorter stretch of approximately 1,000 positions was used (data not shown), we included the shorter sequences for the photosynthetic strains MKAa2 and IRBG 230 (55) in the analysis and found both strains belonging to the same small group. It is clear that this photosynthetic cluster, including *B. denitrificans*, is distinct from *B. japonicum*, *B elkanii*, and other *Bradyrhizobium* sp. strains related to both these species. The photosynthetic cluster would seem about equally distant from *B. japonicum*, the photosynthetic species *R. palustris*, the nitrifying genus *Nitrobacter*, and the pathogenic genus *Afipia* and slightly more distant still from *B. elkanii* (Fig. 3).

The ARDRA technique confirmed that all *Aeschynomene* isolates clustered on the *Bradyrhizobium* phylogenetic branch (Fig. 2) and showed that the majority of the photosynthetic strains formed a distinct sublineage (sublineage A), related to *B. japonicum* (sublineage B) at a correlation coefficient of 86%; both clusters are related to the *B. elkanii* cluster (sublineage C) at a correlation coefficient of 80%.

rRNA-based phylogenetic investigations have shown that the genus *Bradyrhizobium* is closely related to *R palustris*, a photosynthetic bacterium able to grow photoautotrophically under anaerobic conditions (29, 57). This would suggest that *Bradyrhizobium* may have evolved from photosynthetic free-living bacteria by the acquisition of symbiotic functions. Most bradyrhizobia are root symbionts living in a soil-root environment where they are not exposed to significant levels of light. As a consequence of low selection pressure, photosynthetic function may have been lost during evolution from a free-living existence to a symbiotic one. In the particular case of stem nodule symbionts, however, the ancestral trait of photosynthesis may have been retained since remaining genetic information for heterotrophic photosynthesis could still be a selective advantage in both free-living and symbiotic states. The natural habitat of stem-nodulated legumes is restricted to tropical waterlogged or very humid, nitrogen- and carbon-deficient soils. In waterlogged soil or on the plant surface, bacterial photosynthesis may sustain better growth and survival of bacteria and give a competitive advantage for stem nodulation. In symbiosis, bacterial photosynthesis may allow more efficient interaction by reducing the need of the microsymbiont for carbon. Our simultaneous observation in the same *Bradyrhizobium* phylogenetic group of photosynthetic characteristics and specific nodulation abilities supports the hypothesis that a branch of ancestral photosynthetic bacteria has adapted to the particular stem-nodulated *Aeschynomene* environment through acquisition of specific symbiotic functions and conservation of photosynthetic characteristics. However this remains speculative and, alternatively, the possibility that symbiotic bradyrhizobia acquired photosynthetic genes by lateral transfer cannot be excluded. Phylogenetic studies of nodulation and photosynthetic genes may elucidate the origin of these genes. Further investigation is needed to evaluate the role of bacterial photosynthesis in the symbiotic interaction and to evaluate whether preservation of photosynthetic functions reflects an adaptation to the stem-nodulated *Aeschynomene* environment.

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ARTICLE 12

Hannibal L, Lorquin J, Angles d'Ortoli N, Garcia N, Chaintreuil C, Masson-Boivin C, Dreyfus B. et **Giraud E.**

Isolation and characterization of canthaxanthin biosynthesis genes from the photosynthetic *Bradyrhizobium* sp. strain ORS278.

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Isolation and Characterization of Canthaxanthin Biosynthesis Genes from the Photosynthetic Bacterium *Bradyrhizobium* sp. Strain ORS278

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A carotenoid biosynthesis gene cluster involved in canthaxanthin production was isolated from the photosynthetic *Bradyrhizobium* sp. strain ORS278. This cluster includes five genes identified as *crtE*, *crtY*, *crtI*, *crtB*, and *crtW* that are organized in at least two operons. The functional assignment of each open reading frame was confirmed by complementation studies.

Bradyrhizobium strains isolated from *Aeschynomene* stem nodules are photosynthetic (4; see reference 6 for a review), which is a rare trait in *Rhizobium* bacteria. These strains exhibit a photoheterotrophic and strictly aerobic photosynthesis (6; A. Vermeglio, personal communication). In culture, most of these stem isolates show the same pink coloration, while a few strains produce orange pigmentation (12, 16). Pigment analyses showed that bacteriochlorophyll and spirilloxanthin, two pigments of the light harvesting system, are common to all of these photosynthetic *Bradyrhizobium* strains, whereas orange strains produce an additional bicyclic carotenoid, canthaxanthin (4,4′-diketo-β-carotene) (12). This was the first report on the presence of this carotenoid in photosynthetic bacteria. *Bradyrhizobium* sp. strain ORS278 produces the highest quantity of canthaxanthin of all tested photosynthetic bacteria; canthaxanthin represents 85% of its total carotenoid content (12).

Contrary to anaerobic purple phototrophic bacteria, aerobic phototrophic bacteria synthesize an unusually diverse variety of carotenoids, including photosynthetic carotenoids such as spirilloxanthin or spheroidenone, and often a large amount of bicyclic carotenoid (β-carotene and hydroxyl derivatives) (24; see reference 26 for a review). These carotenoids were shown to not be bound to the photosynthetic apparatus of these aerobic bacteria (17, 27) and their function is still unclear—they could have a protective role against photo-oxidative damage, as already observed for several carotenoids (19, 25).

Synthetic canthaxanthin is applied for both direct and indirect food coloring (10, 23). In cosmetology and pharmacology, it is also combined with β-carotene for use as a dermal photoprotector (8). Canthaxanthin is, therefore, a pigment of high economic value, but its level in *Bradyrhizobium* sp. strain ORS278 (1.43 mg/g of dry cell weight) remains insufficient for this organism to be a realistic candidate for natural canthaxanthin production (12). However, it could be possible to enhance the production of canthaxanthin by cloning carotenoid biosynthesis genes of this strain.

In this paper, we describe the cloning and characterization of the canthaxanthin gene cluster of *Bradyrhizobium* sp. strain ORS278.

Isolation of a carotenoid gene cluster. The genes *crtB* and *crtI*, encoding, respectively, phytoene synthase and phytoene desaturase, two enzymes involved in the initial steps of carotenoid biosynthesis (Fig. 1), have been isolated and characterized in various microorganisms (1, 9, 11, 14, 15, 21). In all of these cases, these genes were found to be adjacent and oriented in the same direction. Comparison of the deduced amino

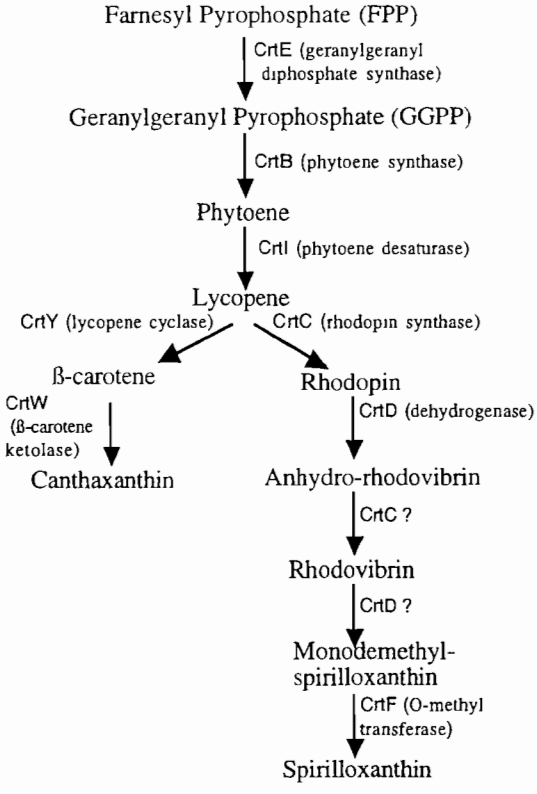

FIG. 1. Scheme of the canthaxanthin (15) and spirilloxanthin (20) biosynthesis pathways.

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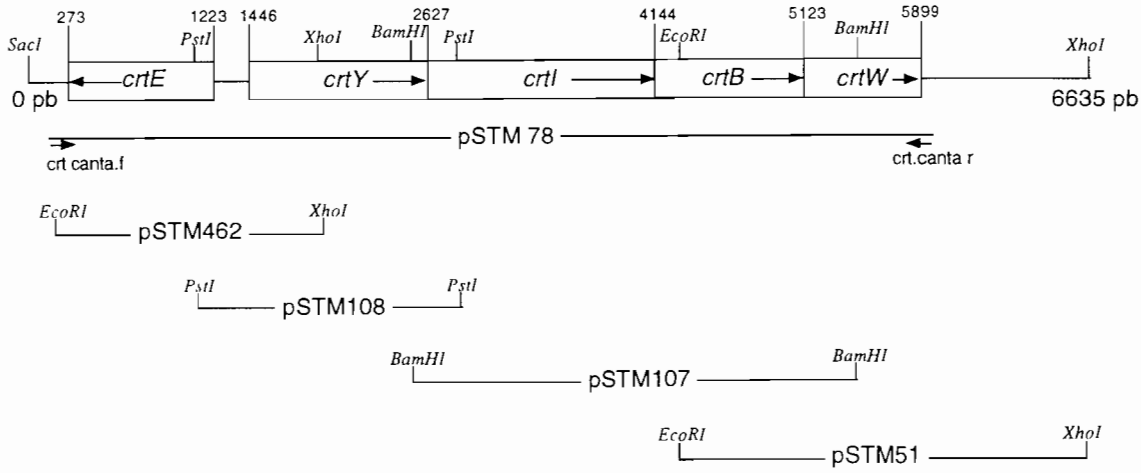

FIG 2 Organization of the canthaxanthin biosynthesis gene cluster of *Bradyrhizobium* sp strain ORS278 and locations of various subcloned fragments The restriction fragments are inserted into pUC18 (pSTM108, pSTM107, and pSTM51) or pUC19 (pSTM462), the *crt* genes are transcribed from the *lac* promoter of the vector In the plasmid pSTM78, the insert was obtained by Long PCR using the primers Crt canta f (5′-GCAACCGGTACCCGAGTTAATTCGCTCGGAATG-3′) and Crt canta r (5′-ATGGTGAAGCTTATGCGGCAGCGGGTTTAGTC-3′) and was cloned into pGEM-T (Promega) In pSTM78, the *crtY*, *crtI*, *crtB*, and *crtW* genes are under *lac* promoter control

acid sequences of the CrtI and CrtB proteins from *Erwinia uredovora*, *Erwinia herbicola*, *Flavobacterium* sp. strain ATCC 21588, *Rhodobacter sphaeroides*, and *Agrobacterium aurantiacum* revealed well-conserved domains at the C-terminal end of CrtI (LVGAGTHPG) and in the central region of CrtB (QLTNIARD). These motifs were chosen for designing the degenerated primers CrtIf (5′-GTNGGNGCRGGCACNCA YCC-3′) and CrtBr (5′-TCGCGRGCRATRTTSGTSARRTG-3′). PCR amplification was performed with a Perkin-Elmer model 2400 thermocycler in a 50-μl (total volume) reaction mixture containing 100 ng of strain ORS278 genomic DNA, each deoxynucleotide triphosphate (200 μM), primers (0.8 μM each), $MgCl_2$ (1.5 mM), 1.25 U of *Taq* DNA polymerase (Promega, Charbonières, France), and the buffer supplied with the enzyme. A touchdown PCR (3) was done as follows: initial denaturation at 94°C for 5 min followed by 20 cycles consisting of a 30-s denaturation at 94°C, 30 s at an annealing temperature of 60 to 50°C, and a 1-min primer extension at 72°C, followed by 15 cycles consisting of a 30-s denaturation at 94°C, 30 s at an annealing temperature at 50°C, and a 1-min primer extension at 72°C. After the final elongation step at 72°C for 7 min, the amplified 620-bp fragment obtained (probe A) was purified by a Wizard procedure and was ligated into a pGEM-T vector (Promega). The ABI Prism BigDye Terminator Cycle Sequence Kit (Applied Biosystems, Foster City, Calif.) was used to sequence the cloned PCR product with the universal oligonucleotides M13 forward and M13 reverse. Sequencing reactions were analyzed on an Applied Biosystems model 310 DNA sequencer. The sequence of the amplified 620-bp fragment was highly similar to known CrtB sequences at the amino acid level.

Two specific primers, CrtIBfow.ORS278 (5′-ATTCGCAGC GGCTCGAAGAG-3′) and CrtIBrev.ORS278 (5′-GATCGCC GACATCATCACGC-3′), based on the sequence of the amplified DNA fragment, were designed for PCR screening of a library of the ORS278 strain constructed with the SuperCos I cosmid vector kit (Stratagene, La Jolla, Calif.), as instructed by the manufacturer. Four positive clones were isolated and confirmed by Southern blot analysis by using the 620-bp fragment as a probe. Clone pSTM73, containing an insert of approximately 35 kb, was used to characterize this *crt* gene cluster.

Structure of the canthaxanthin *crt* gene cluster. A 6.5-kb region in the inserted DNA fragment of the pSTM73 cosmid, showing a positive hybridization signal to probe A, was sequenced and analyzed as shown in Fig. 2. This nucleotide sequence had five open reading frames (ORFs) encoding proteins with similarity to known Crt enzymes (Fig. 3). Based on sequence similarity (45% amino acid identity with CrtY of *E. herbicola*), one of these ORFs was assigned to a *crtY* gene which encodes lycopene cyclase, a key enzyme that converts lycopene into the cyclic carotenoid β-carotene. Another ORF was similar in sequence to a *crtW* gene encoding a β-carotene ketolase that synthesizes canthaxanthin from β-carotene via echinenone (15). This indicated that we had isolated a *crt* gene cluster involved in canthaxanthin biosynthesis. Four of the five ORFs, identified as *crtY*, *crtI*, *crtB*, and *crtW*, were found to be clustered in this order in the same orientation, whereas the ORF *crtE* preceded these four but was oriented in the opposite direction (Fig. 2). The *crtY*, *crtI*, and *crtB* genes are closely linked physically; i.e., the stop codons of *crtY* and *crtI* overlap the start codon of the following ORF, suggesting that these

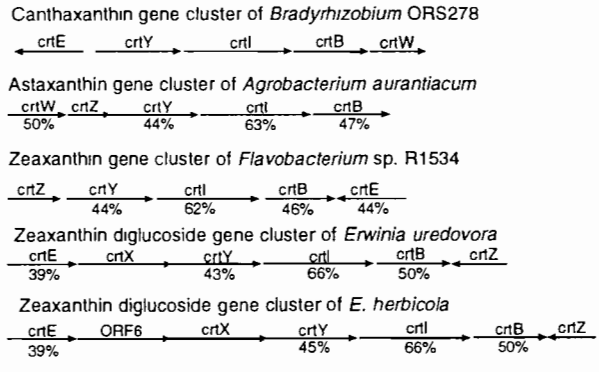

FIG 3. Comparison of the organization of the cyclic carotenoid gene clusters of *Bradyrhizobium* sp strain ORS278, *A. aurantiacum* (15), *Flavobacterium* sp strain R1534 (21), *E uredovora* (14), and *E. herbicola* (9). Arrows represent the orientations of ORFs The percentage values below the genes indicate the percentages of amino acid identity compared to *Bradyrhizobium* sp. strain ORS278.

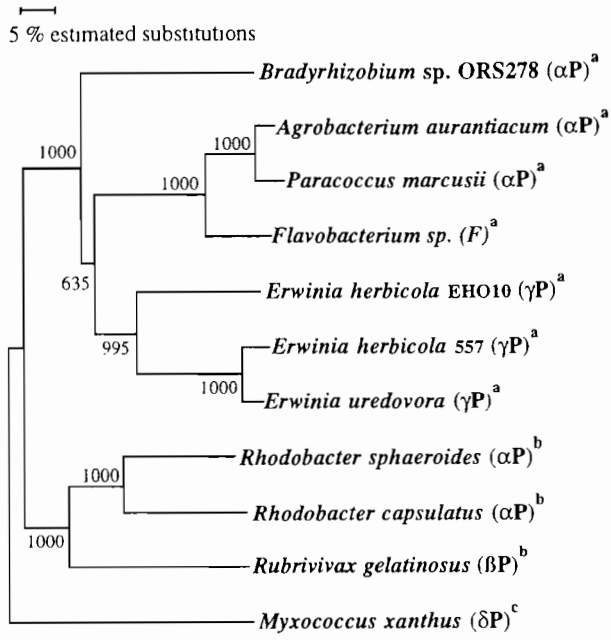

FIG 4. Phylogenetic tree based on the CrtB sequences and constructed by using the neighbor-joining method (22) Bootstrap values (5), expressed as percentages of 1,000 replications, are given at the branching points P, *Proteobacteria*, F, *Flavobacteria*, a, bicyclic carotenoid; b, acyclic carotenoid; c, monocyclic carotenoid GenBank accession numbers are as follows: AF218415, *Bradyrhizobium* sp strain ORS278, D58420, *A aurantiacum*, Y15112, *Paracoccus marcusii*, U62808, *Flavobacterium* sp , M87280, *E herbicola* EHO10, M90698, *E herbicola 557*, D90087, *E uredovora*, AF195122, *R sphaeroides*; *X52291*, *Rhodobacter capsulatus*, *U87626*, *Rubrivax gelatinosus*, Z211955, *Myxococcus xanthus*

genes are translationally coupled (18). Note that the *crtY*, *crtI*, and *crtB* genes always occurred in this order and were oriented in the same direction in the other cyclic carotenoid biosynthesis clusters described previously (see Fig. 3).

Phylogenetic trees were constructed with available CrtI and CrtB sequences. The CrtB proteins (Fig. 4) and the CrtI proteins (data not shown) formed two distinct clusters which do not correlate with the taxonomical position of the strains, but rather with the nature of the carotenoid (cyclic or noncyclic) synthesized under the control of *crtI* and *crtB*. The fact that α- and γ-*Proteobacteria* and *Flavobacteria* group in the same cluster (Fig. 4) suggests that lateral gene transfer has occurred between these phylogenetically unrelated bacteria. Moreover, the fact that strain ORS278 genes are clustered with *crtI* and *crtB* genes from nonphotosynthetic strains producing cyclic carotenoids rather than with photosynthetic strains producing photosynthetic carotenoids raises the question of whether an additional copy of the *crtI* and *crtB* genes involved in the biosynthesis of spirilloxanthin does exist. The *crtC* and *crtD* genes, which were reported to be involved in the biosynthesis of spirilloxanthin from lycopene (20), have just been isolated in another cosmid which did not overlap the pSTM73 cosmid (E. Giraud and B. Dreyfus, unpublished data). We are currently investigating if another copy of the *crtI* and *crtB* genes is physically linked to these *crtC* and *crtD* genes, as has been found in photosynthetic bacteria (1, 11).

Carotenoid production in *Escherichia coli* transformants. *E coli* transformants carrying the entire *crt* gene cluster of canthaxanthin from strain ORS278, cloned in pGEMT (pSTM78) or SuperCosI (pSTM73), did not produce any carotenoids (Table 1), suggesting that these genes are not expressed or that their products are not functional in *E. coli*. Misawa et al. (15) constructed *E. coli* transformants which accumulate each precursor of the zeaxanthin biosynthesis pathway by introducing various combinations of *E. uredovora crt* genes. To check the functionality of the different ORFs identified in strain ORS278, we complemented several carotenoid-accumulating *E. coli* transformants with plasmids carrying various *crt* genes of strain ORS278 and analyzed carotenoids synthesized by high-pressure liquid chromatography (Table 1). The conditions were as follows: 5-μm Hypersil C_{18} column (250 by 4.6 mm; Alltech, Templemars, France), eluent of acetonitrile-methanol-isopropanol (85/10/5, vol/vol/vol), flow rate of 1 ml/min, and detection at 470 nm (450 nm for β-carotene). Peaks were compared and coeluted with standard compounds then identified by their visible spectra and partition coefficients (12).

When plasmid pSTM78 carrying the complete *crt* cluster

TABLE 1. Analysis of carotenoids accumulated in *E. coli* transformants carrying various combinations of *crt* genes from *E. uredovora* and *Bradyrhizobium* sp strain ORS278[a]

E coli host strain characteristics		*E coli* transformant characteristics after complementation	
Plasmid (*crt* genes of *E uredovora* carried)	Carotenoid accumulated[c]	Plasmid introduced[d] (*crt* genes of ORS278 carried)	Carotenoid accumulated[e,f]
None	–[g]	pSTM73 (*crtE crtY crtI crtB crtW*)	–
None	–	pSTM78 (*crtE crtY crtI crtB crtW*)	–
pACCRT-E[b] (*crtE*)	GGPP	pSTM78 (*crtE crtY crtI crtB crtW*)	Canthaxanthin (100%) [95.4]
pSTM420 (*crtI crtB crtY*)	–	pSTM462 (*crtE*)	β-Carotene (98%), nic[h] (2%)
pACCRT-E[b] (*crtE*)	GGPP	pSTM107 (*crtI crtB*)	Lycopene (100%)
pACCRT-EB[b] (*crtE crtB*)	Phytoene	pSTM107 (*crtI crtB*)	Lycopene (100%)
pACCRT-EIB[b] (*crtE crtI crtB*)	Lycopene	pSTM108 (*crtY*)	β-Carotene (100%)
pACCRT-EIBY[b] (*crtE crtI crtB crtY*)	β-Carotene	pSTM51 (*crtW*)	Canthaxanthin (90%) [800], echinenone (2%), nic (8%)

[a] Transformants were grown in Luria-Bertani medium for 36 h in the presence of ampicillin (50 μg/ml), chloramphenicol (30 μg/ml), and 0.125 mM isopropyl-1-thio-β-D-galactopyranoside The carotenoids were extracted according to the method of Lorquin et al. (12).

[b] The plasmids used were described by Misawa et al (15) Plasmid pSTM420 was obtained after deletion by *Sal*I digestion of the *crtE* gene from the plasmid pACCRT-EIBY.

[c] The carotenoids found in *E. coli* host strains are in accordance with the findings of Misawa et al (15)

[d] Details on insertion of the various constructed plasmids are presented in Fig 2

[e] The percentage of the accumulated carotenoid of the total carotenoid content is indicated in parentheses

[f] In square brackets, total (*cis* plus *trans*) canthaxanthin level is indicated in micrograms per gram of dry cell weight

[g] –, carotenoids not detected

[h] nic, nonidentified compound

of *Bradyrhizobium* sp. strain ORS278 was introduced into the *E. coli* transformant that had accumulated geranylgeranyl pyrophosphate (GGPP) as a result of the presence of the *crtE* gene of *E. uredovora*, the new transformant obtained was shown to accumulate canthaxanthin. This result indicates that the *crtY*, *crtI*, *crtB*, and *crtW* genes are functional and allow the production of canthaxanthin in *E. coli*. Nevertheless, the amount of canthaxanthin produced remains lower than in the wild-type strain ORS278. When plasmid pSTM462 carrying the *crtE* gene of *Bradyrhizobium* sp. strain ORS278 under the *lac* promoter was introduced into an *E. coli* transformant containing the *crtI*, *crtB*, and *crtY* genes of *E. uredovora*, the new transformant accumulated β-carotene, showing the functionality of the *crtE* gene.

In this study, we cloned and characterized all of the *crt* genes of *Bradyrhizobium* sp. strain ORS278 necessary for canthaxanthin biosynthesis. This is the first report of a cyclic carotenoid biosynthesis gene cluster in a photosynthetic bacterium. It would be interesting to determine the genetic links of this canthaxanthin *crt* gene cluster to the photosynthetic gene cluster. In *Bradyrhizobium* sp. strain ORS278, canthaxanthin production is stimulated by light (13), suggesting that the expression of canthaxanthin biosynthesis genes is regulated by photoinduction, as already reported for other pigments in different organisms (2, 7). Production of this pigment could be optimized by identifying the signal transduction system controlling canthaxanthin biosynthesis. However, characterization of the entire *crt* gene cluster necessary for canthaxanthin biosynthesis already provides a basis for the construction of a recombinant strain that could overproduce this carotenoid

Nucleotide sequence accession number. The DNA sequence obtained in this study has been deposited in the GenBank database under accession no. AF218415.

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ARTICLE 13

Chaintreuil C, **Giraud E**, Prin Y, Lorquin J, Gillis M, De Lajudie P, Dreyfus B.

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Photosynthetic Bradyrhizobia Are Natural Endophytes of the African Wild Rice *Oryza breviligulata*

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We investigated the presence of endophytic rhizobia within the roots of the wetland wild rice *Oryza breviligulata*, which is the ancestor of the African cultivated rice *Oryza glaberrima*. This primitive rice species grows in the same wetland sites as *Aeschynomene sensitiva*, an aquatic stem-nodulated legume associated with photosynthetic strains of *Bradyrhizobium*. Twenty endophytic and aquatic isolates were obtained at three different sites in West Africa (Senegal and Guinea) from nodal roots of *O. breviligulata* and surrounding water by using *A. sensitiva* as a trap legume. Most endophytic and aquatic isolates were photosynthetic and belonged to the same phylogenetic *Bradyrhizobium/Blastobacter* subgroup as the typical photosynthetic *Bradyrhizobium* strains previously isolated from *Aeschynomene* stem nodules. Nitrogen-fixing activity, measured by acetylene reduction, was detected in rice plants inoculated with endophytic isolates. A 20% increase in the shoot growth and grain yield of *O. breviligulata* grown in a greenhouse was also observed upon inoculation with one endophytic strain and one *Aeschynomene* photosynthetic strain. The photosynthetic *Bradyrhizobium* sp. strain ORS278 extensively colonized the root surface, followed by intercellular, and rarely intracellular, bacterial invasion of the rice roots, which was determined with a *lacZ*-tagged mutant of ORS278. The discovery that photosynthetic *Bradyrhizobium* strains, which are usually known to induce nitrogen-fixing nodules on stems of the legume *Aeschynomene*, are also natural true endophytes of the primitive rice *O. breviligulata* could significantly enhance cultivated rice production.

Bacteria of the genera *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Allorhizobium*, *Mesorhizobium*, and *Azorhizobium*, commonly known as rhizobia, have great environmental and agricultural importance because their symbioses with legumes are responsible for most of the atmospheric nitrogen fixed on earth. These microorganisms are soil bacteria able to elicit the formation of new organs, called nodules, on most species of the family *Fabaceae* and on the nonleguminous *Parasponia*, in which they reduce atmospheric nitrogen to ammonia to the benefit of the host plant. In the absence of leguminous plants, populations of rhizobia are commonly found in soils where they can survive saprophytically. Recently, the natural habitat of rhizobia was extended to a third niche, the roots of gramineous plants. *Rhizobium leguminosarum* bv. *trifolii* was shown to be naturally present inside the roots of rice grown in rotation with clover in Egypt, without forming any nodule-like structure (44). Such habitats inside roots of cereals and grass plants have already been identified as an important reservoir for various N_2-fixing endophytic bacteria, known as plant growth-promoting rhizobacteria (PGPR), such as *Acetobacter diazotrophicus* (11, 36) and *Herbaspirillum seropedicae* (5, 7, 22) in sugar cane, *Azoarcus* spp. (34, 35) in Kallar grass (*Leptochloa fusca*), and *Azospirillum* spp. (6) in maize and rice. Like most of these PGPR on their homologous gramineous host plants, *R. leguminosarum* was shown to efficiently promote rice growth after field inoculation (44).

Rice (*Oryza sativa* L.) is the most important food crop in developing countries. The high-yielding rice varieties of the "Green Revolution" have resulted in large increases in rice production but require large amounts of nitrogen fertilizers, which contribute to nitrate contamination of soils and groundwater supplies, often leading to health hazards and environmental pollution. Therefore, endophytic rhizobia, known to directly supply biologically fixed nitrogen to the legume plant, may also have great potential to improve sustainable rice production.

In order to discover other natural rice-rhizobium associations, we screened sustainable primitive rice production systems where N fertilizer has never been applied. In Africa, the wetland wild rice *Oryza breviligulata*, which is the ancestor of the African cultivated rice *Oryza glaberrima* (38), has been harvested and consumed in the Sahelian and Sudanian regions for more than 10,000 years. *O. breviligulata* grows spontaneously in temporary ponds, wetland plains, and river deltas of the semiarid and semihumid regions of Africa. It grows in 0.5- to 1.5-m-deep water, where it floats and forms numerous nodal and aquatic roots. This primitive rice species is frequently found growing in association with several aquatic legumes belonging to the genera *Aeschynomene* and *Sesbania* (1, 14, 19). Among these aquatic species, *Aeschynomene indica* and *Aeschynomene sensitiva* form stem nodules with photosynthetic *Bradyrhizobium* strains (26), and *Sesbania rostrata* forms stem nodules with *Azorhizobium caulinodans* (29). We therefore screened three *O. breviligulata* growing sites in West Africa (two sites in Senegal and one in Guinea) for natural associations of photosynthetic bradyrhizobia and/or azorhizobia with the rice roots.

MATERIALS AND METHODS

Isolation of endophytic rhizobia from wild rice. Young nodal roots of 10 different *O. breviligulata* plants per site were collected at the flowering stage in wild-paddy fields of Senegal and Guinea (West Africa) where the water was 30 cm to 1 m deep. Roots were cleaned thoroughly with tap water, rinsed with sterile deionized water, drained on absorbent paper, and cut into 3- to 5-cm-long

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TABLE 1. Photosynthetic endophytic and aquatic strains used in the present study

Bacterial strain[a]	Source	Geographic origin	Nodulation specificity[b]			Crt type[c]
			Aeschynomene elaphroxylon (group I)	*A. afraspera* (group II)	*A. sensitiva* (group III)	
ORS402	Rice root	Joal (Senegal)	0	0	E	W
ORS404	Water	Joal (Senegal)	0	0	E	DP
ORS405	Water	Joal (Senegal)	0	0	I	W
ORS406	Water	Joal (Senegal)	0	0	I	W
ORS408	Water	Joal (Senegal)	0	0	E	DP
ORS2005	Rice root	Joal (Senegal)	0	0	E	O
ORS2006	Rice root	Joal (Senegal)	0	0	E	DP
ORS2007	Rice root	Joal (Senegal)	0	0	E	O
ORS2008	Rice root	Joal (Senegal)	0	0	E	DP
ORS2009	Rice root	Joal (Senegal)	0	0	E	DP
ORS2010	Water	Joal (Senegal)	0	0	E	DP
ORS2011	Rice root	Joal (Senegal)	0	0	E	O
ORS2012	Rice root	Joal (Senegal)	0	0	E	DP
ORS2013	Water	Joal (Senegal)	0	0	E	DP
ORS2014	Rice root	Joal (Senegal)	0	0	E	O
ORS2019	Water	Joal (Senegal)	0	0	E	O
STM478	Rice root	Kapachez (Guinea)	0	0	E	DP
STM481	Rice root	Kapachez (Guinea)	0	0	E	DP
STM482	Rice root	Kapachez (Guinea)	0	0	E	DP
STM515	Rice root	Kapachez (Guinea)	0	0	E	DP

[a] Designations ORS, collection of the Institut de Recherche pour le Développement, Montpellier, France; STM, collection of the Laboratoire des Symbioses Tropicales et Méditerranéennes, Montpellier, France. Bacterial strains listed here are new isolates

[b] 0, no nodulation, I, ineffective root nodulation, E, effective root nodulation.

[c] Crt, carotenoid, W, white (strain lacking Bchl *a* and carotenoid); DP, dark pink; O, orange.

sections. Five grams of sections from each individual collected plant was then transferred to a sterile 250-ml Erlenmeyer flask containing 50 ml of sterile water, shaken for 15 min, and washed twice in 50 ml of sterile distilled water. Sections were then aseptically transferred to another sterile 250-ml Erlenmeyer flask and surface sterilized as follows. They were placed in 96% ethanol for 1 min, washed with sterile distilled water, then sterilized with 0.1% $HgCl_2$ for 5 min, and washed six times with sterile distilled water. As a control to check for superficial contamination, for each individual plant, 200 μl of water from the final rinse was inoculated on plates containing tryptone-glucose-yeast extract agar (TY) medium (Difco, Detroit, Mich.). No individual root sample resulting in contamination on TY plates was retained for isolation of endophytic rhizobia. Sections were aseptically crushed in a Waring blender containing 100 ml of sterile water, and 0.5 ml of the root homogenate was then aseptically inoculated into 4-day-old young seedlings of *A. sensitiva*, *S. rostrata*, and *Acacia albida* grown in Gibson tubes (41) (see below). Enumeration of rhizobia from surface-sterilized root homogenates, non-surface-sterilized roots, or rice field water was performed by the plant infection most-probable-number (MPN) technique as described by Brockwell (10).

Legume cultivation and rhizobium isolation from nodules. Seeds of *A. sensitiva*, *A. indica*, *Aeschynomene afraspera*, *S. rostrata*, and *F. albida* were scarified, surface sterilized for 45 min with concentrated sulfuric acid, and rinsed six times with sterile distilled water. Seeds were incubated to germinate in sterile petri dishes on 0.8% agar yeast extract-mannitol (YEM) medium (41). After 24 to 48 h, seeds showing no contamination were transferred to Gibson tubes containing nitrogen-free Jensen seedling slant agar (23) and grown under continuous light (20 W/m^2) at 28°C. Two days later, legume species (eight replications for each treatment) were aseptically inoculated with rhizobial cultures or root homogenate. Three-week-old nodulated plants were collected, and nodules were picked, washed, and surface sterilized by immersion in $HgCl_2$ (0.1%) for 5 min. From this stage, the nodules were manipulated aseptically. Nodules were rinsed six times in sterile distilled water and then crushed in a drop of sterile water, and the suspension was streaked onto YEM plates. After 1 week of incubation at 28°C under aerobic conditions, colonies were checked for purity by repeated streaking on YEM medium and by microscopic examination of living cells. Isolates were checked for nodulation on their original host plants.

Bacterial strains and culture growth conditions. The bacterial isolates used in this study are listed in Table 1. All *Bradyrhizobium* strains were maintained on YEM medium. All strains were stored at −80°C on YEM medium adjusted to 20% (vol/vol) glycerol. Representative and type strains of *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* and various clusters of *Bradyrhizobium* strains previously described (13, 15, 26, 27) were included in this study (Table 2).

Rice cultivation and nitrogen fixation activity. Wild rice seeds were hulled, placed in 96% ethanol for 15 min, rinsed twice with sterile water, sterilized for 25 min in 0.1% $HgCl_2$, and rinsed six times in sterile distilled water. Seeds were germinated in the dark at 30°C for 2 days on plates containing semisolid TY medium (0.8% agar-agar [wt/vol]). Plantlets with a 1- to 2-cm-long root showing no contamination were soaked for 5 min in an axenic mid-log-phase rhizobial culture. The inoculated rootlet was packed between two sterile filter papers in a large petri plate containing Jensen nitrogen-free medium. The plants were grown at 28°C for 3 to 4 weeks in growth chambers. Nitrogen-fixing activity was estimated by measurement of the acetylene-reducing activity of roots by the method of Hardy et al. (21).

Greenhouse rice inoculation experiments. Two-week-old rice plantlets grown on sterile sand in a nursery and previously inoculated were transplanted into pots containing 5 kg of local unsterilized sandy soil (psamment) with 93% sand. The first control consisted of noninoculated, non-N-fertilized plantlets, and the second control comprised noninoculated but N-fertilized plantlets that received 5 mM KNO_3 added in two equal doses 15 days after transplanting and at the mid-tillering stage. The experiment was arranged in a randomized design with six replicates for each treatment. Pots were watered with tap water and, every 2 weeks, with nitrogen-free Jensen's plant growth medium, to maintain a 2-cm waterhead above the soil surface. Experiments were performed in a greenhouse between June and October at 27 to 34°C with a day length of about 12 h. All *O. breviligulata* plants were harvested 115 days after transplanting. Grain, shoot, and leaf yields were evaluated.

Microscopic examination of endophytes within rice roots. At days 15 and 30 after inoculation with a *lacZ*-tagged strain of ORS278 (strain M10) (E. Giraud and L. Hannibal, personal communication), roots were histochemically treated as described earlier by Boivin et al. (8) and screened under an Olympus SZH10 stereomicroscope. Control roots inoculated with the wild-type strain ORS278 and the *Bradyrhizobium* strain Aust13c originally isolated from *Acacia mangium* (20) were also examined. Transverse sections (40 μm thick) were made using a Leica VT1000S Vibratome. Microscopic preparations were examined with an Olympus Provis microscope.

Photosynthetic pigment determination. Cultures were grown at 30°C for 7 days under aerobic conditions on a cycle of 15 h of light and 9 h of darkness. Bacteriochlorophyll *a* (Bchl *a*) was extracted under dim light with cold acetone-methanol (7:2, vol/vol) at 4°C for 30 min (25). The supernatant was analyzed using a Beckman DU40 spectrophotometer. Absorption spectra were obtained by scanning over a wavelength range from 350 to 800 nm. Carotenoids were further purified and analyzed by high-pressure liquid chromatography (HPLC) or thin-layer chromatography (TLC) as previously described (25).

16S amplified ribosomal DNA restriction analysis (ARDRA). Strains were grown at 28°C for 5 days on YEM. Total DNA was purified by a slightly modified technique from Pitcher et al. (31) described by Doignon-Bourcier et al. (13). The primers and the general conditions for 16S rRNA gene PCR amplification were those described by Doignon-Bourcier et al. (13) except that amplifications were carried out in a Gene Amp PCR System 2400 (Perkin-Elmer). Endonuclease restriction of PCR products was performed with *Hinf*I, *Dde*I, *Mwo*I, *Alu*I, and *Hha*I. Restricted DNA was then analyzed by horizontal agarose gel electrophoresis using Metaphor agarose (FMC Bioproducts). Clustering was obtained by restriction profile analyses using the GelCompar 4.2 software package (40). The Dice coefficient (Tol, 0.3%; Opt, 0.50%; minimum area, 0.0%) and UPGMA (unweighted pair group method using average linkage) clustering were used.

Analysis of the 16S rRNA genes. We amplified the nearly full-length 16S rDNA of six photosynthetic strains by using the two specific oligonucleotide

TABLE 2. Reference strains used in this study[a]

Bacterial strain	LMG no	Original host plant	Geographic origin	Reference or source	Crt type spectrum[b]
Photosynthetic *Bradyrhizobium* spp.					
ORS266	15442	*Aeschynomene tambacoundensis*	Senegal	26	LP
ORS276	12185	*Aeschynomene sensitiva*	Senegal	26	
ORS278	12187	*Aeschynomene sensitiva*	Senegal	26	O
ORS279	12188	*Aeschynomene sensitiva*	Senegal	26	
ORS280	15411	*Aeschynomene indica*	Senegal	26	
ORS287	15378t1	*Aeschynomene afraspera*	Senegal	26	LP
ORS288	12189	*Aeschynomene afraspera*	Senegal	26	
ORS292	12205	*Aeschynomene sensitiva*	Senegal	26	
ORS294	12192	*Aeschynomene sensitiva*	Senegal	26	LP
ORS296	12194	*Aeschynomene sensitiva*	Senegal	26	
ORS297	12195	*Aeschynomene sensitiva*	Senegal	26	LP
ORS299	15399	*Aeschynomene sensitiva*	Senegal	26	
ORS306	11797	*Aeschynomene indica*	Senegal	1	LP
ORS308	15401	*Aeschynomene afraspera*	Senegal	3	LP
ORS324	8295	*Aeschynomene afraspera*	Senegal	2	LP
ORS331	11799	*Aeschynomene tambacoundensis*	Senegal	2	LP
ORS337	8299t2	*Aeschynomene afraspera*	Senegal	3	
ORS342	15383	*Aeschynomene indica*	Senegal	26	
ORS344	12198	*Aeschynomene indica*	Senegal	26	
ORS348	12200	*Aeschynomene* sp	Senegal	26	
ORS352	15384	*Aeschynomene afraspera*	Senegal	1	LP
ORS359	12201	*Aeschynomene sensitiva*	Senegal	26	LP
ORS362	10305	*Aeschynomene afraspera*	Senegal	2	
ORS364	11802	*Aeschynomene nilotica*	Senegal	2	LP
ORS368	15387	*Aeschynomene indica*	Senegal	26	
ORS371	11804	*Aeschynomene indica*	Senegal	26	DP
ORS377	15420	*Aeschynomene elaphroxylon*	Senegal	26	W
ORS378	15421	*Aeschynomene elaphroxylon*	Senegal	26	W
ORS379	15422	*Aeschynomene elaphroxylon*	Senegal	26	W
ORS381	15423	*Aeschynomene elaphroxylon*	Senegal	26	W
ORS384	15390	*Aeschynomene indica*	Senegal	26	LP
ORS385	15391	*Aeschynomene indica*	Senegal	26	
ORS386	11815	*Aeschynomene indica*	Senegal	26	LP
ORS392	12205	*Aeschynomene indica*	Senegal	26	O
ORS400	12207	*Aeschynomene indica*	Senegal	26	
ORS29	15165	Unknown	Senegal	13	
ORS31	15167	*Indigofera tinctoria*	Senegal	13	
ORS87	15177	*Tephrosia purpurea*	Senegal	13	
ORS90	R-2196	*Tephrosia purpurea*	Senegal	13	
ORS110	10666	*Acacia albida*	Senegal	15	
ORS122	10678	*Acacia albida*	Senegal	15	
ORS123	10679	*Acacia albida*	Senegal	15	
ORS124	10680	*Acacia albida*	Senegal	15	
ORS127	10683	*Acacia albida*	Senegal	15	
ORS133	10689	*Acacia albida*	Senegal	15	
ORS140	10696	*Acacia albida*	Senegal	15	
ORS167	10710	*Acacia albida*	Senegal	15	
ORS169	10712	*Acacia albida*	Senegal	15	
ORS175	10718	*Acacia albida*	Senegal	15	
ORS182	10721	*Acacia albida*	Senegal	15	
ORS183	10722	*Acacia albida*	Senegal	15	
ORS523	R-2194	*Indigofera senegalensis*	Senegal	13	
ORS520	R-2192	*Indigofera senegalensis*	Senegal	13	
ORS935	15269	*Rhynchosia minima*	Senegal	13	
ORS937	15271	*Rhynchosia minima*	Senegal	13	
ORS976	R-2197	*Indigofera senegalensis*	Senegal	13	
ORS979	15275	*Indigofera senegalensis*	Senegal	13	
ORS980	15276	*Indigofera senegalensis*	Senegal	13	
ORS984	15279	*Indigofera senegalensis*	Senegal	13	
ORS1217	15301	*Indigofera senegalensis*	Senegal	13	
ORS1218	R-2200	*Indigofera senegalensis*	Senegal	13	
ORS1219	15302	*Indigofera senegalensis*	Senegal	13	
ORS1228	15304	*Indigofera astragalina*	Senegal	13	
ORS1810	15242	*Crotalaria lathyroides*	Senegal	13	
ORS1813	15244	*Crotalaria hyssopifolia*	Senegal	13	
ORS1814	15245	*Crotalaria hyssopifolia*	Senegal	13	

Continued on following page

TABLE 2—*Continued*

Bacterial strain	LMG no	Original host plant	Geographic origin	Reference or source	Crt type spectrum[b]
ORS1819	15249	*Crotalaria retusa*	Senegal	13	
ORS1820	15250	*Indigofera hirsuta*	Senegal	13	
ORS1823	15367	*Indigofera hirsuta*	Senegal	13	
ORS1824	15153	*Indigofera hirsuta*	Senegal	13	
ORS1844	15266	*Chamaecrista* sp.	Senegal	13	
ORS1848	15373	*Indigofera hirsuta*	Senegal	13	
ORS1849	15374	*Indigofera hirsuta*	Senegal	13	
ORS1898	15699	*Tephrosia bracteolata*	Senegal	13	
ORS1903	15702	*Tephrosia villosa*	Senegal	13	
BTAi1	11795	*Aeschynomene indica*	United States	16	LP
BR3606	9959	*Acacia mollissima*	Brazil	27	
BR3621	9966	*Acacia mangium*	Brazil	27	
CB756	8319	*Macrotylona africanum*	Zimbabwe		
NZP2192	6161	*Lotus corniculatus*	New Zealand		
NZP2309	6128	*Lotus pedunculatus*	Australia		
NZP2314	6129	*Lotus pedunculatus*	Australia		
MSDJ718		*Lupinus luteus*	France		
TAL309	8316				
Nonphotosynthetic *Bradyrhizobium* spp.					
ORS272	15408	*Aeschynomene schimperi*	Senegal	26	
ORS313	15416	*Aeschynomene afraspera*		2	
ORS317	15147	*Aeschynomene afraspera*		2	
ORS354	15424	*Aeschynomene afraspera*		2	
Bradyrhizobium japonicum					
USDA135	8321	*Glycine max*	United States		
USDA110		*Glycine max*	United States		
MAR1589	14312	*Arachis hypogaea*			
NZP5533	6136	*Glycine max*	United States		
NZP5549T	6138T	*Glycine max*	Japan		
Bonnier 3 1	4252	*Glycine max*			
1BOa2	4262	*Albizia julibrissin*			
Bradyrhizobium liaoningense					
SFI 2062	18231	*Glycine max*	China	43	
SFI 2281	18230	*Glycine max*	China	43	
Bradyrhizobium elkanii					
NZP 5532	6135	*Glycine max*	United States	24	
NZP 5531T	6134T	*Glycine max*	United States	24	

[a] Photosynthetic strains are in boldface. Designations: ORS, collection of the Institut de Recherche pour le Développement, Montpellier, France; LMG, collection of bacteria of the Laboratorium voor Microbiologie, University of Ghent, Ghent, Belgium; BR, strain from the Centro Nacional de Pesquisa em Biologia do Solo (CNPBS), Seropédica, and Empresa Brasiliera de Pesquisa Agropequaria (EMBRAPA), Rio de Janeiro, Brazil; INPA, National Institute of Amazonia Research, Manaus, Brazil; NZP, Culture Collection of the Department for Scientific and Industrial Research, Biochemistry Division, Palmerston North, New Zealand; USDA, U S Department of Agriculture, Beltsville, Md.; TAL, Nitrogen Fixation in Tropical Agricultural Legumes (NifTAL), University of Hawaii, Paia; MSDJ, Institut National de la Recherche Agronomique (INRA), Microbiologie des Sols, Dijon, France; MAR, Soil Productivity Research Laboratory, Marondera, Zimbabwe; SFI, Soils and Fertilizers Institute, Chinese Academy of Agricultural Sciences, Beijing, People's Republic of China

[b] Crt, carotenoid; W, white (strain lacking Bchl *a* and carotenoid); DP, dark pink; O, orange; LP, light pink.

primers FGPS6, 5′-GGAGAGTTAGATCTTGGCTCAG-3′ (sense; positions 6 to 27 by *Escherichia coli* numbering), and FGPS1509, 5′-AAGGAGGGGATC CAGCCGCA-3′ (antisense; positions 1540 to 1521 by *E. coli* numbering), according to the method of Normand et al. (30). PCR products were purified with a QIAquick Gel extraction Kit (Qiagen, Courtaboeuf, France) The ABI Prism BigDye Terminator Cycle sequence kit (Applied Biosystems, Foster City, Calif.) was used to directly sequence the purified PCR product Sequencing reactions were analyzed on an Applied Biosystems model 310 DNA sequencer.

DNA sequence analysis. The six 16S rDNA sequences obtained were compared to the GenBank database by using the algorithm BLASTN (4) to identify the most similar 16S rDNA sequences. An alignment was performed, using the PILEUP program (12), with a set of sequences of representatives of the most closely related genera identified A phylogenetic tree was constructed by the neighbor-joining method (37), and a bootstrap confidence analysis was performed on 1,000 replicates to determine the reliability of the tree topology obtained (18).

Nucleotide sequence accession numbers. The GenBank accession numbers for the 16S rRNA gene sequences of strains ORS2005, ORS2006, ORS2011, ORS2012, STM481, and ORS278 are AF230718, AF230719, AF230720, AF230721, AF239254, and AF239255, respectively.

RESULTS

Isolation of endophytic rhizobia from wild rice. Nitrogen-fixing nodules were formed within 2 weeks on the roots of *A. sensitiva*, used as a trap legume host and previously inoculated with crushed surface-sterilized nodal roots of *O. breviligulata*. Thirteen endophytic rhizobial isolates were independently obtained from these *Aeschynomene* nodules (Fig. 1 and Table 1). Of these, nine isolates (ORS2005, ORS2006, ORS2007, ORS2008, ORS2009, ORS2011, ORS2012, ORS2014, and ORS402) were obtained from two distant temporary ponds of the coastal region of Joal and Nianing in Senegal. The other four isolates (STM478, STM481, STM482, and STM515) were obtained from deep-water wild rice growing in the Baga wetland coastal region of Guinea, located 600 km south of the Senegalese sampling sites. Both of the other legumes used as

trap hosts for rice endophytic rhizobia, *S. rostrata* and *A. albida*, failed to develop any nodules upon inoculation with crushed rice roots.

Isolation of aquatic rhizobia from rice field water. In order to compare the endophytic and aquatic rhizobia, water samples from the two wild rice growing sites in Senegal were also tested for the presence of free rhizobia (Fig. 1). Six isolates (ORS404, ORS405, ORS406, ORS408, ORS2010, and ORS2013) were obtained from nodules of *A. sensitiva* previously inoculated with water samples. One isolate (ORS2019) was obtained by direct streaking of the water sample onto YEM plates and was selected as a result of its pink pigmentation.

Symbiotic characterization of endophytic and aquatic rhizobial isolates. To determine to which specificity group the endophytic and aquatic rhizobia belonged, we performed root inoculation tests with representative plants of different nodulation groups as defined by Alazard (1). The 20 endophytic and aquatic isolates showed a very specific nodulation pattern: they nodulated only *A. sensitiva* and *A. indica*, two species belonging to the most specific *Aeschynomene* cross-inoculation group (Table 1). Nodules were effective, except for those induced by both aquatic isolates, ORS405 and ORS406, that did not fix nitrogen. None of the isolates induced nodulation on *A. afraspera*, which belongs to another *Aeschynomene* cross-inoculation group, or on *A. albida*, a nonspecific host known to be nodulated by a broad range of typical promiscuous "cowpea" *Bradyrhizobium* strains.

Photosynthetic properties of endophytic and aquatic rhizobia. All isolates developed typical slow-growing *Bradyrhizobium* colonies on YEM plates. With the exception of isolates ORS402, ORS405, and ORS406, which formed white colonies, all isolates synthesized pink or orange pigments on yeast-mannitol solid or liquid culture medium (Table 1), particularly when exposed to light, suggesting the presence of carotenoids and photosynthetic pigments (17, 25). All pigmented isolates produced an absorbance peak at 770 nm, characteristic of Bchl *a*, and peaks around 400 to 500 nm, corresponding to carotenoids. No Bchl *a* or carotenoids were observed in isolates ORS402, ORS405, and ORS406.

All pigmented isolates (ORS404, ORS408, ORS478, ORS481, ORS482, ORS515, ORS2005, ORS2006, ORS2007, ORS2008, ORS2009, ORS2010, ORS2011, ORS2012, ORS2013, ORS2014, and ORS2019), and one nonpigmented isolate (ORS402) gave a fragment of the expected size (926 bp) using the primers defined from the *pufLM* sequence of *Bradyrhizobium* sp. strain ORS278 as described by Molouba et al. (26). The sequences of the amplified products obtained with two representative endophytic isolates (ORS2005 and ORS2011) were homologous to those of the *pufLM* genes. No PCR products were obtained with isolates ORS405 and ORS406.

ARDRA. We also performed ARDRA to compare the 20 new isolates with *Bradyrhizobium* strains representative of the ARDRA groups previously described (13, 26). The results are shown as a dendrogram in Fig. 2. At a mean Dice similarity coefficient (S_D) of 76%, we distinguished three main clusters. All the endophytic and aquatic isolates, except ORS405 and ORS406, grouped in the same major cluster together with photosynthetic *Bradyrhizobium* sp. strains from *Aeschynomene*. The two other main clusters, containing the type strains of the three named *Bradyrhizobium* species, were previously described by Doignon-Bourcier et al. (13).

16S rRNA gene sequence analysis. We determined the 16S rRNA gene sequences of the representative endophytic strains ORS2005, ORS2006, ORS2011, ORS2012, and STM481. All five sequences were identical and were 2, 6, 9, and 10 bp different from those of ORS278, USDA4377, BTAi1, and *Blastobacter denitrificans* strain LMG8443, respectively. A phylogenetic tree was constructed to determine the position of these isolates among other *Bradyrhizobium* strains (Fig. 3). All the endophytes formed a separate branch together with the photosynthetic *Bradyrhizobium* sp. strains ORS278, BTAi1, and USDA 4377 from *Aeschynomene* and the aquatic strain *B. denitrificans* (LMG8443). This grouping was supported by a bootstrap value of 100% and was distinct from the five other well-separated clusters including, respectively, *B. japonicum*, *B. elkanii*, *Rhodopseudomonas palustris*, *Nitrobacter winogradskyi*, and *Afipia felis* (Fig. 3).

Numeration of endophytic and aquatic rhizobia. We found a population density of $\sim 5.0 \times 10^6$ endophytic rhizobia per g (fresh weight) of surface-sterilized rice roots. When the roots were not surface sterilized, the total population density of rhizobia reached $\sim 2.5 \times 10^7$ rhizobia per g of roots. Populations of aquatic rhizobia able to nodulate *Aeschynomene* were evaluated as $\sim 1.1 \times 10^2$ rhizobia per ml of water.

Inoculation with endophytic rhizobia of the wild rice grown in a greenhouse. Sixteen rice endophytic and aquatic isolates and two photosynthetic *Bradyrhizobium* strains from *Aeschynomene* were inoculated on rice plants grown in sandy soil without added N fertilizer. After growth, the dry weights of shoots and leaves and of grains were determined; results are shown in Table 3. The endophytic isolate ORS2011 and the *Aeschynomene* photosynthetic *Bradyrhizobium* strain ORS278 produced the highest effects of inoculation, giving 20% increases in both shoot and grain yields. Inoculation with most other isolates, including strain BTAi1, resulted in dry weights of shoots and leaves similar to those of the N-fertilized control. With half of these isolates, however, no inoculation effect was observed on grain yields, which remained very low, as in the noninoculated, nonfertilized control.

Colonization and infection of wild rice roots by photosynthetic rhizobia. Because the photosynthetic *Bradyrhizobium* strain ORS278 was the strain with the highest inoculation effect on the growth and grain yield of rice grown in a greenhouse, we used a marked derivative of ORS278 (strain M10) to trace the infection process in rice roots. Strain M10 was constructed independently from this work by insertion of a *lacZ* reporter gene in the photosynthetic *puf* gene (Giraud and Hannibal, personal communication). The *lacZ* reporter gene was shown to be strongly expressed under the culture conditions we used for rice growth. The wild-type strain ORS278 and the nonphotosynthetic *Bradyrhizobium* strain Aust13c from *A. mangium* were used as controls. Fifteen days after inoculation of *O. breviligulata* with strain M10, histochemical staining of β-galactosidase activity revealed a strong bacterial colonization of the root cap which exhibited a dense blue staining (Fig. 4A). This colonization was particularly dense at the surface of the mucilage drop that covers the root apex (Fig. 4B). Senescent root cells, sloughed off the root cap, often exhibited a hairbrush shape (Fig. 4C), due to polar attachment of bacterial cells covering their surfaces. Polar attachment also occurred with the wild-type strain ORS278. By contrast, despite colonization of the mucilage, we never observed such polar attachment with the nonphotosynthetic *Bradyrhizobium* strain Aust13c (data not shown). Thirty days after inoculation, clusters of sloughed root cap cells were still observed far from the root tip, embedded in mucilage and densely colonized by *Bradyrhizobium* (Fig. 4E). An intense bacterial colonization of the root surface originated from these clusters (Fig. 4E). Bacteria appeared lined up along the borders of adjacent epidermal cells (Fig. 4G). Bacterial invasion developed deeper in the intercellular spaces as revealed by cross sections (Fig. 4H). A few intracellular infections were also regularly observed in epi-

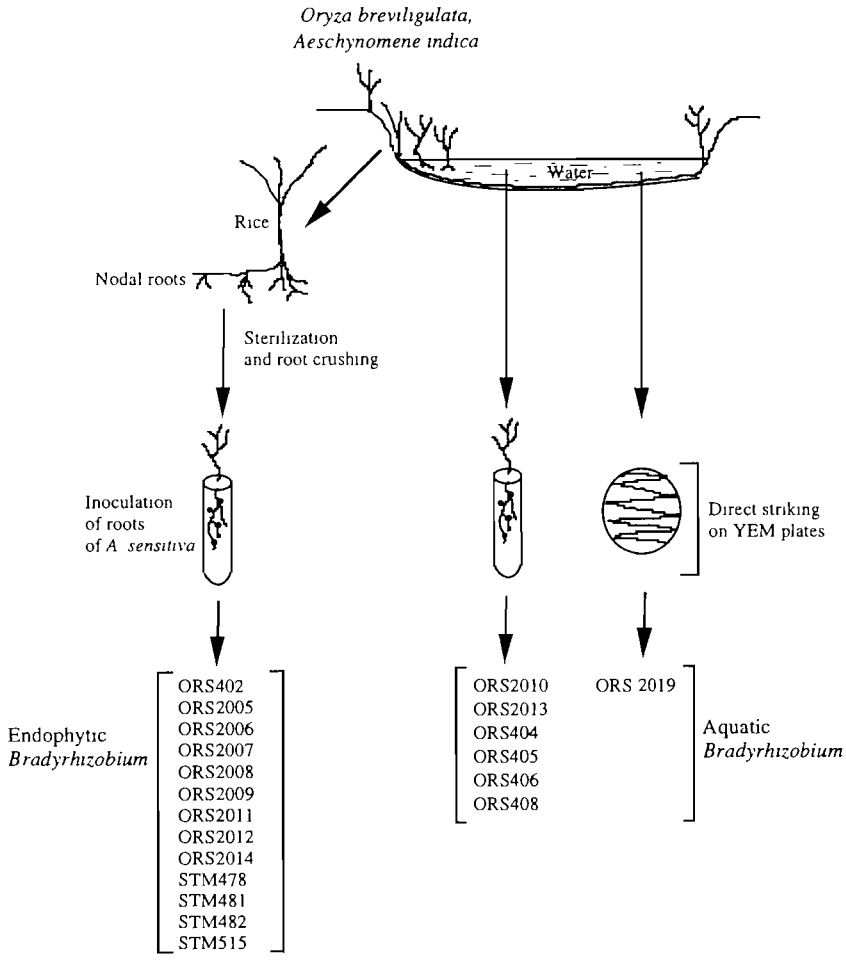

FIG 1 Isolation of endophytic and aquatic *Bradyrhizobium* strains

dermal cells filled with bradyrhizobia (Fig. 4I). In the zone of emergence of lateral roots, dense bacterial proliferation was observed in the fissures caused by protruding lateral roots (Fig. 4D), where bacterial proliferation commonly reached four to five cell layers deep (Fig. 4F).

Nitrogen-fixing activity in the rice rhizosphere. A low but significant level of nitrogen-fixing activity, as measured by the acetylene reducing activity, was detected (1.7 nmol of C_2H_4/h/plant) on 4-week-old rice plants artificially grown in growth chambers in large petri dishes and inoculated with the photosynthetic strain ORS278. No acetylene reducing activity was detected with the noninoculated rice control.

DISCUSSION

Photosynthetic *Bradyrhizobium* strains are known to specifically induce nitrogen-fixing nodules on stems and roots of aquatic legumes of the genus *Aeschynomene* (26). We report here for the first time that these photosynthetic symbiotic bacteria also form a natural endophytic association with the wild rice species *O. breviligulata*. The surfaces and interiors of the rice roots, which we found densely colonized by *Aeschynomene*-nodulating *Bradyrhizobium* strains, therefore appear in nature as unexpected niches, much more abundant than root and stem nodules of the aquatic legumes. Photosynthetic *Bradyrhizobium* strains were also directly isolated from water, thus confirming their aquatic character (9), which could result in a bacterial selective advantage in the absence of rice or legume hosts. Yanni et al. (44) previously reported endophytic association between another rhizobial species, *R. leguminosarum* bv. *trifolii*, and rice grown in Egypt in rotation with the legume *Trifolium alexandrinum*. Our results with *O. breviligulata* thus suggest that rhizobia could be usual endophytic bacteria of various primitive and cultivated rice species.

Growth stimulation of different cereals such as wheat or rice following seed inoculation with nonphotosynthetic rhizobia such as *R. leguminosarum* (44) or *Azorhizobium* (42) strains has been previously reported. We therefore conducted greenhouse experiments to measure plant growth responses to inoculation with photosynthetic endophytic isolates. Inoculation with both the photosynthetic isolate ORS2011 and the *Aeschynomene* photosynthetic strain ORS278 resulted in statistically significant increases in shoot and grain yields, indicating their potential ability to enhance rice production. As we detected a nitrogen-fixing activity in inoculated rice plants, nitrogen fixation could contribute to this plant growth-promoting response. Nitrogenase activity had previously been detected in wheat inoculated with *Azorhizobium* strain ORS571, but only when succinate was added to the plant growth medium (42). Photosynthetic *Bradyrhizobium* strains are known to fix nitrogen under free-living conditions (2). However, we cannot conclude that the low N_2 fixation activity detected would support on its own a major role in the increase in shoot and grain yields. It has been shown that rhizobia can secrete indoleacetic acid and

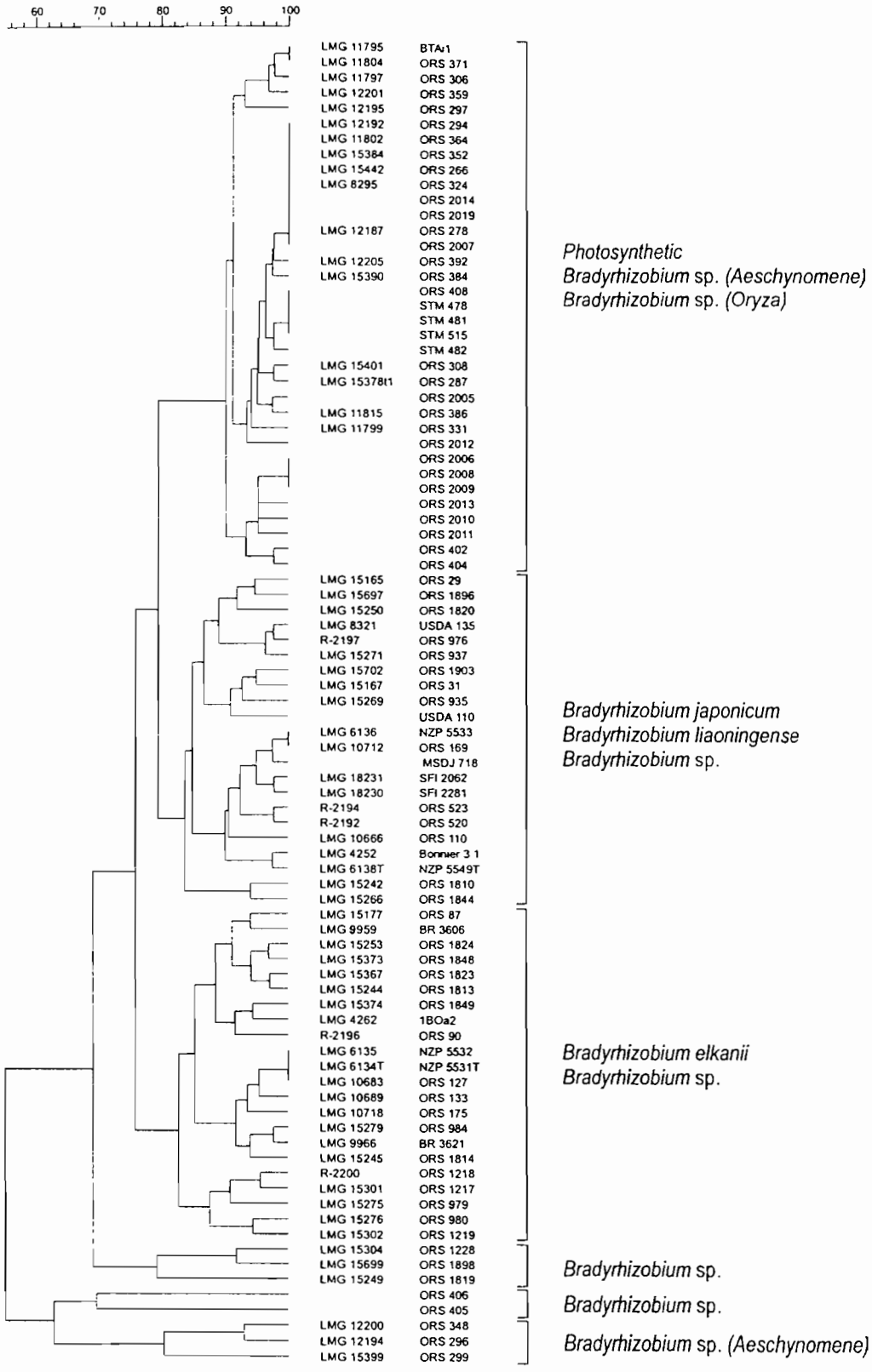

FIG 2 Dendrogram based on UPGMA clustering of Dice correlation values (S_D) of normalized and combined ARDRA patterns of new isolates and reference *Bradyrhizobium* strains obtained with the restriction enzyme combination *Hinf*I, *Dde*I, *Mwo*I, *Alu*I, and *Hha*I. The scale represents S_D values converted to percentages.

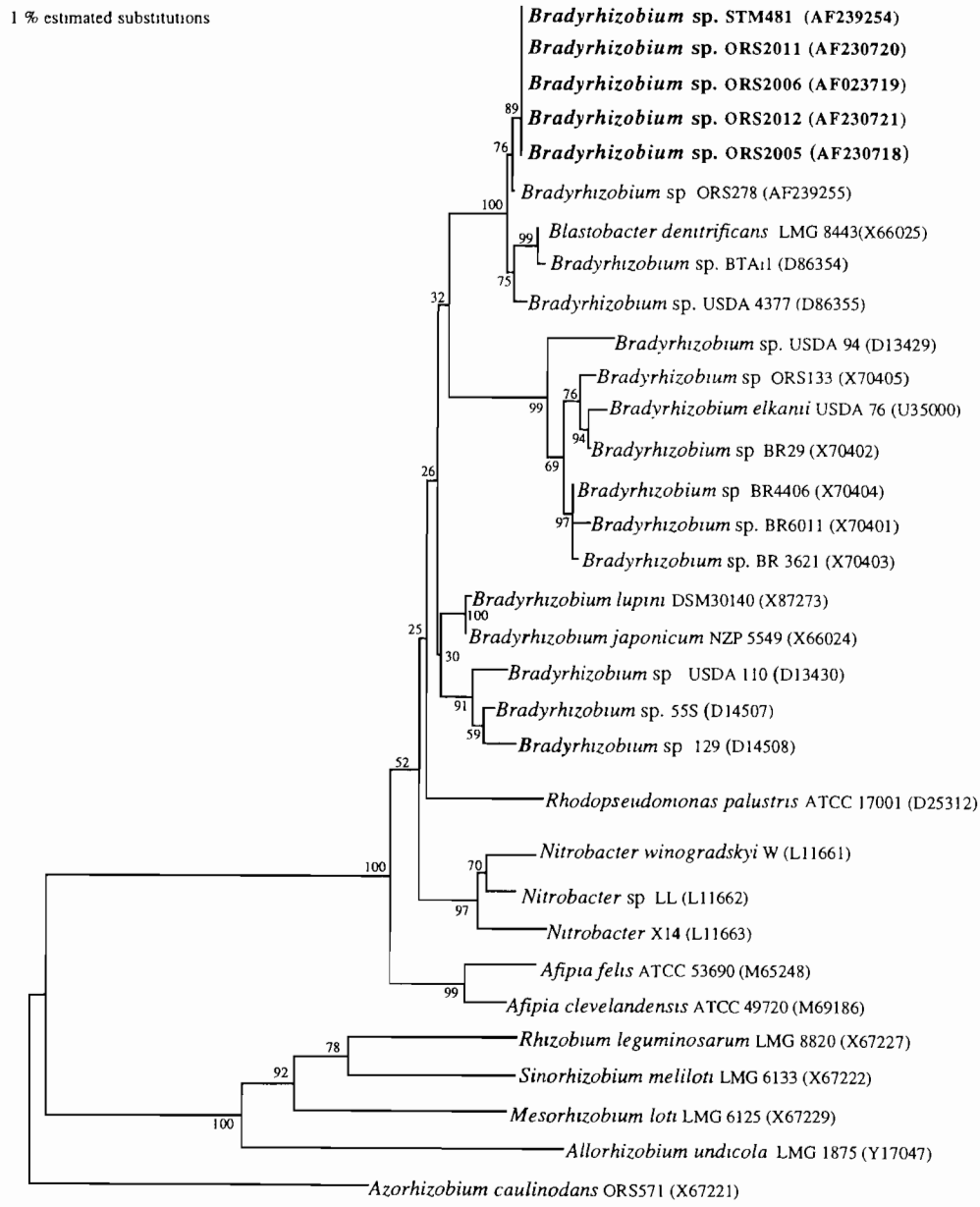

FIG. 3. Neighbor-joining dendrogram of 16S rDNA sequences showing the position of endophytic strains (boldfaced) isolated from wild rice among bradyrhizobia and closely related taxa Bootstrap values, expressed as percentages of 1,000 replications, are given at the branching points. Numbers in parentheses are the accession numbers of the sequences used The bar represents 1 estimated substitution per 100 nucleotide positions.

gibberellic acid phytohormones (44). The free N_2-fixing strains ORS2011 and ORS278 could thus represent an interesting model to determine the respective roles of N_2 fixation and phytohormone production in the significant benefit to rice.

By microscopic examination, we were able to distinguish two main stages of bradyrhizobial colonization of the rice root. The first step was the root cap colonization, followed by a strong bacterial multiplication covering large areas of the root surface. The root cap is known as one of the main sites of polysaccharide secretion by specialized plant cells completely embedded in a mucilage gel-like matrix which has been described as a substrate as well as a niche of proliferation for a wide range of soil microorganisms (32). As the root growth goes on, root cap cells are continuously renewed, and senescent cells are sloughed off along the root. This step, characterized by the polar attachment of the rhizobial cells to the sloughed cells, could be considered characteristic, since a nonphotosynthetic *Bradyrhizobium* strain from *A. mangium* showed no attachment to rice cells. Such oriented attachment has also been observed on the root hairs and root epidermis of rice artificially inoculated with *A. caulinodans* strain ORS571 (33). In *O. breviligulata*, attachment of photosynthetic *Bradyrhizobium* to root cap cells could facilitate the dispersal of the bacteria all along the root. We indeed observed an intense bacterial colonization of large areas of root surface, starting from the sloughed cells acting as an inoculant. Bacteria appeared lined up along the borders of epidermal root cells. Such a preferential location could result from a better bacterial proliferation favored by the presence of intercellular mucilage.

The second step of infection was the intercellular invasion of

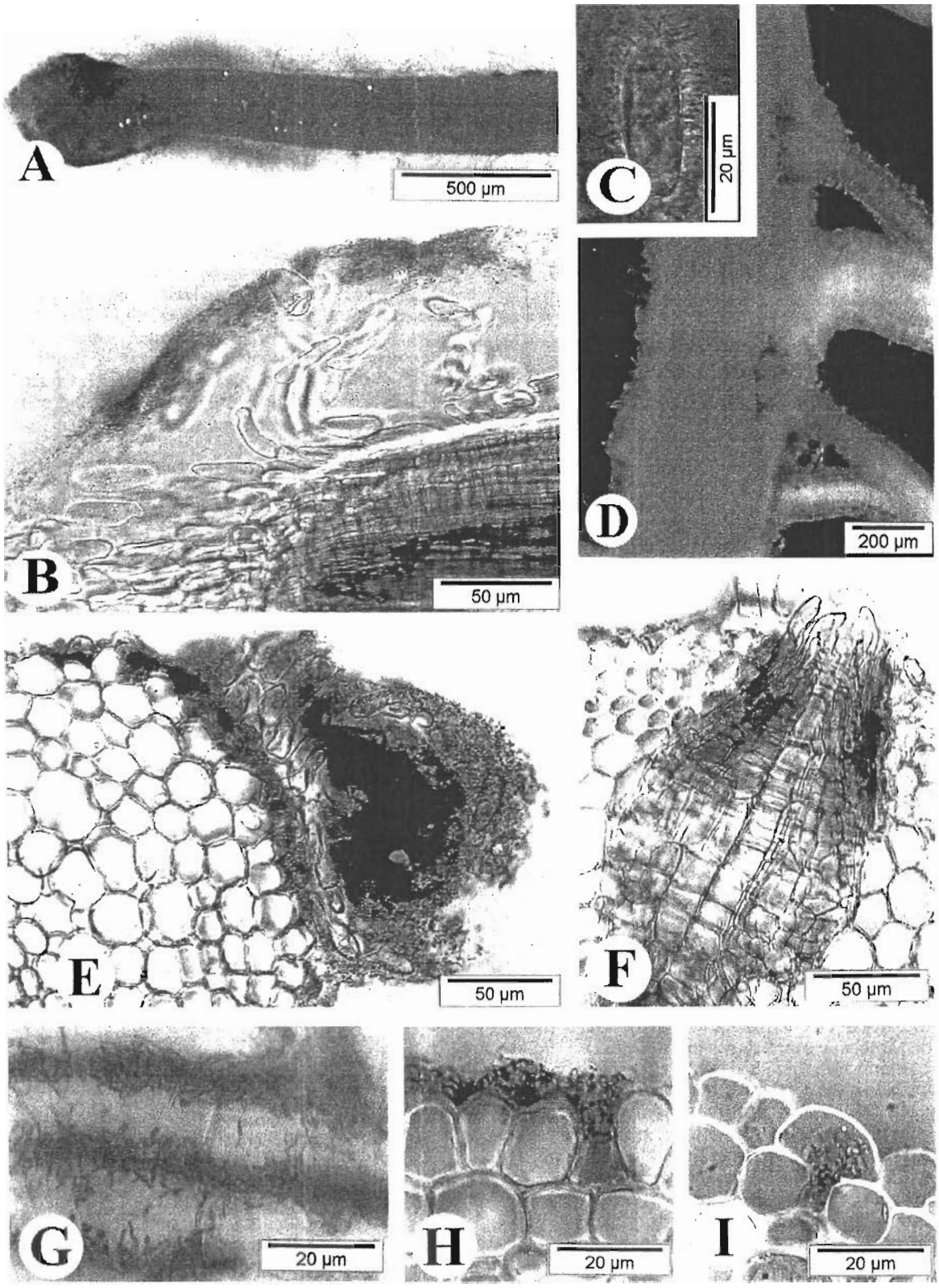

FIG. 4. Stereo- and light microscopic observations of *O. breviligulata* seedlings inoculated with a *lacZ*-tagged *Bradyrhizobium* ORS278 strain (M10). (A to C) Fifteen days after inoculation; (D to I) 30 days after inoculation. (A) Stereomicroscopic observation from the root apex to the root hair zone. An intense blue coloration occurs on the root cap surface, revealing a dense bacterial colonization. (B) Longitudinal section of the root apex showing the dense colonization of the mucilage drop that covers the root cap, viewed by bright-field microscopy. (C) A sloughed root cap covered with *Bradyrhizobium* cells attached in a polar way, observed by phase-contrast microscopy. (D) Colonization of the cracks at the emergence sites of lateral roots, observed by stereomicroscopy. (E) Cross section of the elongation zone of a root with densely colonized root cap remnants, from which the bacteria colonize the root surface, observed by bright-field microscopy. (F) Deep colonization of cracks at the emergence of a lateral roots, as seen in a cross section by bright-field microscopy. (G) Colonization of the root surface along borders of epidermal cells, observed by bright-field microscopy. (H) Cross section of a root with proliferation in the intercellular space between two epidermal cells, observed by bright-field microscopy. (I) Intracellular colonization of an epidermal cell by ORS278, observed by bright-field microscopy.

TABLE 3. Effect of inoculation with endophytic and nonendophytic photosynthetic *Bradyrhizobium* strains on shoot and grain yields of *O. breviligulata* grown in a greenhouse

Strain[a]	Dry wt[b] (g) of	
	Shoots and leaves	Grain
ORS405	19.7†,‡	1.9‡
ORS2019	16.7‡	3.5‡
ORS404	20.7†,‡	4.5‡
ORS2012	21.4†,‡	4.6‡
ORS402	23.0†	4.9‡
ORS408	26.4†	5.0‡
ORS2014	24.3†	6.1‡
ORS2008	23.6†	6.8†,‡
ORS2010	20.5†,‡	7.4†
ORS406	22.4†	7.5†
ORS2005	22.4†	8.1†
ORS2009	23.2†	8.6†
ORS2013	21.8†,‡	8.7†
ORS2007	24.3†	9.4*,†
ORS2006	27.5*,†	9.6*,†
ORS2011	29.7*	10.3*
ORS278	30.1*	12.0*
BTAil	23.4†	7.2†
Control (noninoculated)	16.7‡	5.0‡
Control (N fertilization)	23.0†	8.5†

[a] ORS strains are from the collection of the Institut de Recherche pour le Développement, Montpellier, France

[b] Values with different symbols differ significantly at the $P < 0.01$ level. Analysis of variance was carried out by Fisher's test (39). Six replicates were performed per treatment.

epidermal cells, which constituted the true endophytic stage. Simultaneously with bacterial root surface colonization, numerous lateral roots emerge, producing fissures in the root epidermis and underneath cell layers. These fissures are sites of intense intercellular bacterial proliferation. Bacteria invade the fissure via disjoined epidermis cells and migrate towards deeper layers of cortical cells, between which they form pockets of proliferating bacteria. Such an endophytic stage, in which bacterial cells are densely packed in a confined space, could constitute preferential sites of exchanges between the plant and the bacteria. These large intercellular pockets closely resemble the intercellular infection pockets formed during the early stages of nodulation by rhizobia in aquatic legumes such as *S. rostrata* (28) and *A. afraspera* (3). In these tropical legumes, infection starts intercellularly, directly through "crack entry" at the sites of emerging lateral roots, without formation of infection threads in root hairs as in *Medicago* or *Trifolium*. In *S. rostrata*, infection threads develop ultimately from these pockets and allow the release of the bacteria into the meristematic cells (28). The most primitive infection process is found in *A. afraspera*, where bacteria from the intercellular pockets directly invade the host cell by localized cell wall degradation, without any formation of infection threads (3). Further development of the nodule occurs by repeated division of the infected host legume cells. Interestingly, in *O. breviligulata*, photosynthetic bradyrhizobia are also present intracellularly in cortical cells. However, unlike in *Aeschynomene*, the number of invaded cells remained limited in *O. breviligulata*, and no division of these infected cells was observed. This intracellular invasion could thus be the ultimate stage of rice infection by *Bradyrhizobium*. Nevertheless, the infection process in the monocotyledonous plant *O. breviligulata* by the same photosynthetic bradyrhizobia seems to be very similar to the first stages of infection in the leguminous dicotyledonous plant *Aeschynomene*. Webster et al. (42) showed that colonization of rice and wheat roots by *A. caulinodans* strain ORS571 was *nod* gene independent. With the photosynthetic *Bradyrhizobium* strain ORS278, one hypothesis could also be that, in both *A. sensitiva* and *O. breviligulata*, expression of *nod* genes is not necessary for the first steps of infection involving primitive "crack entry" and direct intercellular invasion. Intracellular invasion in rice has also been reported with the endophytic bacterium *Azoarcus* sp. (34) and with *A. caulinodans* strain ORS571 (33). The reason why intracellular infection does not spread further in rice is not yet elucidated. One hypothesis could be that the endodermis constitutes a thick-walled boundary to infection. Alternatively, rice may lack part of the genetic program necessary for invaded cells to control the intracellular infection and subsequently be capable of repeated divisions. In conclusion, the *O. breviligulata* model, which offers a unique combination of a primitive rice species with primitive "crack entry" rhizobial infection, could represent a major step forward in achieving an efficient nitrogen-fixing endosymbiotic association between rice and rhizobia.

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ARTICLE 14

Giraud E, Hannibal L, Fardoux J, Verméglio A, Dreyfus B.

Effect of *Bradyrhizobium* photosynthesis on stem nodulation of *Aeschynomene sensitiva*.

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Effect of *Bradyrhizobium* photosynthesis on stem nodulation of *Aeschynomene sensitiva*

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Some leguminous species of the genus *Aeschynomene* are specifically stem-nodulated by photosynthetic bradyrhizobia. To study the effect of bacterial photosynthesis during symbiosis, we generated a photosynthesis-negative mutant of the *Bradyrhizobium* sp. strain ORS278 symbiont of *Aeschynomene sensitiva*. The presence of a functional photosynthetic unit in bacteroids and the high expression of the photosynthetic genes observed in stem nodules demonstrate that the bacteria are photosynthetically active during stem symbiosis. Stem inoculation by the photosynthetic mutant gave a 50% decrease in stem-nodule number, which reduced nitrogen fixation activity and plant growth in the same proportion. These results indicate an important role of bacterial photosynthesis in the efficiency of stem nodulation.

Rhizobia interact symbiotically with leguminous plants by inducing nitrogen-fixing nodules on the roots. Some plants of the genus *Aeschynomene*, encountered in waterlogged soils or riverbanks, have the peculiar property of forming stem nodules. This very unusual behavior among leguminous plants is shared only with a few species of the genera *Sesbania, Neptunia,* and *Discolobium* (1). Rhizobia isolated from *Aeschynomene* stem nodules exhibit a property uncommon among other rhizobia of developing a photosynthetic system (2, 3). This was first demonstrated in the case of a strain isolated from *Aeschynomene indica* stem nodules and designated BTAi 1 (2). In *ex planta* culture, BTAi 1 required a photoperiod for production and accumulation of bacteriochlorophyll *a* and exhibited a photoheterotrophic and strictly aerobic photosynthesis (4, 5). In this respect, the photosynthetic activity of the bacteriochlorophyll-containing rhizobia most closely resembles that of the aerobic anoxygenic photosynthetic bacteria (6, 7).

Since the discovery of BTAi 1, several other rhizobia isolated from *Aeschynomene* stem nodules have been reported to produce bacteriochlorophyll *a* (8–13). These photosynthetic *Aeschynomene* rhizobia were shown to form a separate subbranch of the *Bradyrhizobium* rRNA lineage, distinct from *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* (13). All photosynthetic strains were shown to nodulate stem-nodulating *Aeschynomene* species specifically (13), indicating that the photosynthetic character of these rhizobia could play an important role during this unusual symbiosis. The demonstration by Evans *et al.* (4) that illumination of the stem nodules of *A. indica* by far-red light consistently accelerated acetylene reduction and the report of Fleischman and Kramer (3) on the presence of bacteriochlorophyll *a* in bacteroids isolated from stem nodules of *Aeschynomene aspera* provide further evidence for the importance of the photochemical activity of the endophytes in the stem nodules. However, no genetic study has determined the real contribution of the bacterial photosynthesis in the symbiotic interaction and its impact on plant growth.

In purple bacteria, most of the genes required for the formation of the photosystem are clustered in a 45-kb region (14). These include *bch* and *crt* genes coding respectively for the enzymes of the bacteriochlorophyll and carotenoid pathways and the *puf* genes coding for the subunits of the light-harvesting complex (*pufB* and *pufA*) and of the reaction center (RC) complex (*pufL* and *pufM*).

We report here the isolation and characterization of the *puf* genes from a photosynthetic *Bradyrhizobium*, strain ORS278, and reveal the unusual presence of a heme oxygenase gene (*hmuO*) just downstream from the *pufM* gene. ORS278 mutants deleted for heme oxygenase (HmuO) and RC subunit genes were constructed to elucidate the putative role of their products during symbiosis with *Aeschynomene sensitiva*.

Materials and Methods

Bacterial Strains and Growth Conditions. *Bradyrhizobium* sp. strain ORS278 (12), called the wild-type strain throughout this study, and isogenic mutants 278Δ*pufLM* and 278Δ*hmuO* constructed in this work were grown in a modified YM medium (pH 6.8) containing (per liter) 2.5 g of sodium glutamate, 2 g of yeast extract, 0.66 g of K_2HPO_4, 0.05 g of NaCl, 0.1 g of $MgSO_4 \cdot 7H_2O$, 0.04 g of $CaCl_2$, and 0.004 g of $FeCl_3$. Strains were grown aerobically in a gyratory shaker (170 rpm) at 37°C in either complete darkness or continuous white light with a bank of incandescent bulbs or under a light/dark cycle (16 h/8 h). Standard methods were used for *Escherichia coli* growth in Luria–Bertani (LB) medium supplemented with the appropriate antibiotics.

DNA Manipulations. Unless otherwise stated, standard methods for DNA manipulation were used (15). To amplify the *pufL* and *pufM* genes of ORS278 by PCR, degenerate primers were used: pufLf, (5′-TTYTAYGTNGGNTTYTTYGG-3′) and pufMr, (5′-CCCATNGTCCANCGCCARAA-3′). PCR amplification, sequence reactions, and sequence analysis were performed as previously described (16).

Construction and Screening of a Genomic Library of *Bradyrhizobium* sp. Strain ORS278. A genomic library of *Bradyrhizobium* ORS278 was constructed by using the SuperCos I cosmid vector kit (Stratagene). A positive clone, pSTM1, was found by PCR screening using the primers pufL.278f (5′-CACCCATCTCGATTGGGTGTCG-3′) and pufM.278r (5′-CTCCAGCTGCCCATGAAGATCG-3′).

Abbreviations RC, reaction center; HmuO, heme oxygenase

Data deposition The sequence reported in this paper has been deposited in the GenBank database (accession no. AF182374)

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Mapping of *puf* and *hmuO* Transcriptional Start Sites. RNA was prepared as described by Cabanes *et al.* (17). The transcriptional start sites of the *puf* operon and the *hmuO* gene were mapped by primer extension as described by Batut *et al.* (18), using, respectively, the primer pufB.rev (5′-TATGGAATTC-CCTAGCCTCCGATTC-3′) and the primer hmuO.rev (5′-GCCGTTTCGACGTGATTGGAATC-3′).

Construction of *puf* and *hmuO* Mutant Strains. For the construction of a *puf* mutant, a 2.8-kb *Eco*RI–*Bam*HI insert of pSTM1 containing the *pufL* and *pufM* genes of ORS278 was cloned into the *Eco*RI/*Bam*HI sites of pUC18 (Roche, Meylan, France), liberated by *Eco*RI/*Hin*dIII digestion, and cloned into the *Eco*RI/*Hin*dIII sites of pBluescript (SK+) (Stratagene). Subsequently, the 0.8-kb *Bgl*II fragment containing a part of the *pufL* and *pufM* genes was replaced by the 4.7-kb *Bam*HI *lacZ*-Km^r cassette of pKOK5 (19). The resulting 6.7-kb *Bam*HI inserts containing the mutated *pufL* and *pufM* genes were cloned into the pJQ200mp18 suicide vector (20). To mutate the *hmuO* gene, the 1 8-kb *Bgl*II fragment of pSTM1 containing the *hmuO* gene of ORS278 was cloned into the *Bam*HI sites of pJQ200mp18. The 3′ region of the *hmuO* gene was then deleted by *Bam*HI digestion and replaced by the 4.7-kb *Bam*HI *lacZ*-Km^r cassette of pKOK5. The pJQ200 derivatives obtained, which encoded a counterselectable *sacB* marker, were transformed into *E. coli* S17-1 (21) for mobilization into ORS278. After conjugation, colonies grown on YM medium containing kanamycin and nalidixic acid were plated onto YM medium containing 5% sucrose and kanamycin. Sucrose-resistant colonies were screened by PCR to verify that the double-crossover event had occurred. The genomic structure of the mutants was further verified by appropriate Southern blot hybridization of chromosomal DNA.

Preparation of Membranes. *Bradyrhizobium* cells, resuspended in 50 mM Tris·HCl (pH 8), were disrupted by three passages through a French Press at 50 MPa. The suspension was spun for 20 min at 4,000 × *g* to remove the unbroken cells. The supernatant was then spun at 255,000 × *g* for 90 min. The pellet, resuspended in 50 mM Tris·HCl (pH 8), constituted the intracytoplasmic membrane fraction.

Preparation of Endosymbiotic Bacteria. Nodules (5 g) were harvested 5 weeks after inoculation, resuspended with 10 ml of 50 mM KH_2PO_4/Na_2PO_4 buffer (pH 7.4) containing 1 g of polyvinylpyrrolidone, and ground with a mortar and pestle. Debris was removed by centrifugation for 5 min at 1,500 × *g*. Bacteroids were pelleted by centrifugation of the supernatant for 8 min at 8,000 × *g*, washed twice with 50 mM phosphate buffer (pH 7.4) containing 2 mM $MgSO_4$ and 0.3 M sucrose, and resuspended in 1 ml of 50 mM phosphate buffer (pH 7.4).

Absorption and Light-Induced Absorption Change Measurements. Light-induced absorption changes on intact cells were performed as previously described (22). Absorption spectra of bacteroids or membranes isolated from *ex planta* culture of ORS278 were recorded with a Cary 50 probe spectrophotometer.

Plant Tests. *A. sensitiva* seeds were surface-sterilized with concentrated sulfuric acid for 40 min, abundantly rinsed with sterile water, allowed to soak overnight in water, and transferred to plastic pots (8-cm diameter) filled with atapulgite. The plants were grown in a greenhouse at 27–36°C with a relative humidity of 70–80%. Plants were watered regularly and supplied once a week with a plant nutrition solution (23) supplemented with 1 mM KNO_3 until inoculation. After four weeks, the plants were root or stem inoculated. For root inoculation tests, 1 ml of a culture was deposited at the bottom of the plant, whereas stem inoculation was performed by careful painting. The inocula were prepared by growing a culture of either the wild-type strain or the mutant bacterial strains for 3 days, centrifuging the cells, washing them twice and resuspending them in water to obtain an OD of 0.5 at 600 nm. The plants were harvested 5 weeks after inoculation. Nitrogen-fixing activity was estimated on the entire plant by measurement of acetylene reducing activity according to Hardy *et al.* (24), and the dry mass was measured.

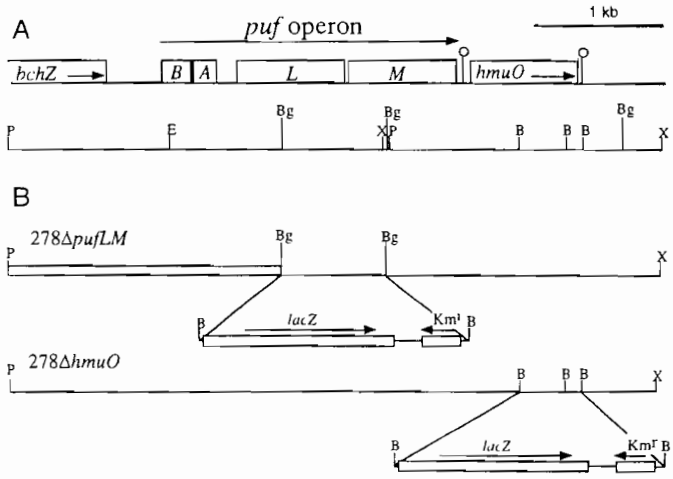

Fig. 1. Physical map of the *Bradyrhizobium* sp. strain ORS278 *puf* region and genetic structure of *puf* and *hmuO* mutations. (*A*) The genes are indicated by open boxes: B, *pufB*; A, *pufA*; L, *pufL*; M, *pufM*. The positions of putative hairpin structures are indicated by open circles. The positions of relevant restriction sites are also shown. P, *Pst*I; X, *Xho*I; E, *Eco*RI; B, *Bam*HI; Bg, *Bgl*II. (*B*) The structures of *puf* and *hmuO* mutations are shown along with the corresponding strain names. Horizontal arrows indicate the orientation of the cassette containing the reporter gene *lacZ* and Km^r.

β-Galactosidase Assays and Light Microscopy. Forty-micrometer-thick sections of fresh nodules were made by using a Leica VT1000S Vibratome and histochemically treated for β-galactosidase staining as described by Boivin *et al.* (25).

Results

Isolation and Characterization of the *puf* Operon of *Bradyrhizobium* Sp. Strain ORS278. PCR amplification using degenerate primers pufLf/pufMr, based on conserved domains of the PufL and PufM proteins (data not shown), gave, with the genomic DNA of the ORS278 strain as template, a fragment of 1,546 bp (probe A), whose sequence was highly similar to those of the *pufL* and *pufM* genes from other photosynthetic bacteria. Subsequently, two specific primers (pufL278f/pufM278r), based on the sequence of the amplified fragment, were designed and used for PCR screening of a genomic DNA library of the ORS278 strain. A positive clone, pSTM1, which contains an insert of approximately 35 kb, was used to characterize the *puf* gene cluster. A 5.1-kb region in the DNA insert of the pSTM1 cosmid, showing positive hybridization to probe A, was sequenced and analyzed, as shown in Fig. 1. This nucleotide sequence had five ORFs, each of which had a putative Shine–Dalgarno box preceding the start codon, ATG. Comparisons with the *puf* genes of other photosynthetic bacteria revealed that four of the five ORFs were *pufB*, *pufA*, *pufL*, and *pufM*, which encode the β and α subunits of the LH1 light-harvesting complex, and the L and M subunits of the RC complex, respectively. PufL and PufM proteins of *Bradyrhizobium* sp. strain ORS278 showed the greatest identity to those of *Rhodopseudomonas palustris* (75% and 69%, respectively) and *Rhodospirillum rubrum* (68% and 69%, respectively).

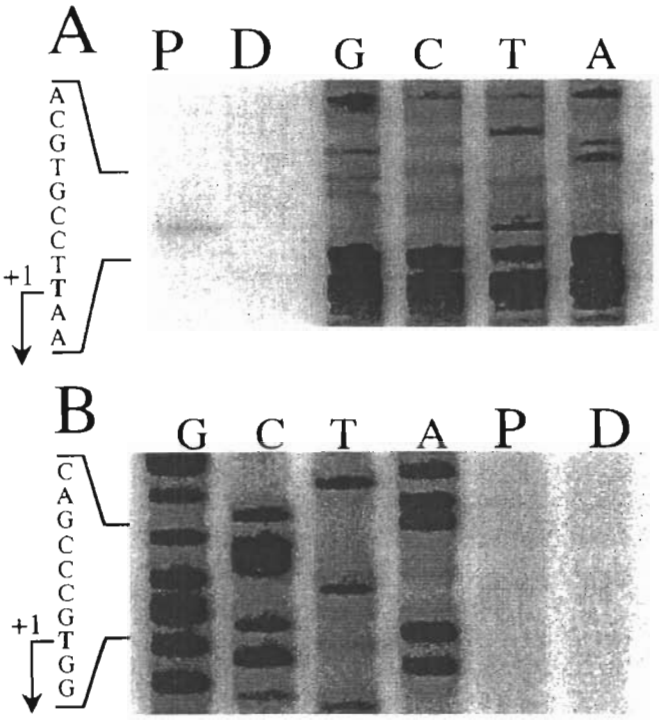

Fig. 2. Determination of the transcriptional start sites of the *puf* operon (*A*) and *hmuO* (*B*). Primer extension analyses were performed with RNAs extracted from ORS278 strain cultured under a dark–light illumination cycle (P) or in darkness (D). As a reference, a DNA sequencing ladder is shown (lanes G, C, T, and A). The sequences were obtained with the same primers used for the primer extensions. The transcription start points (+1) are indicated by arrows.

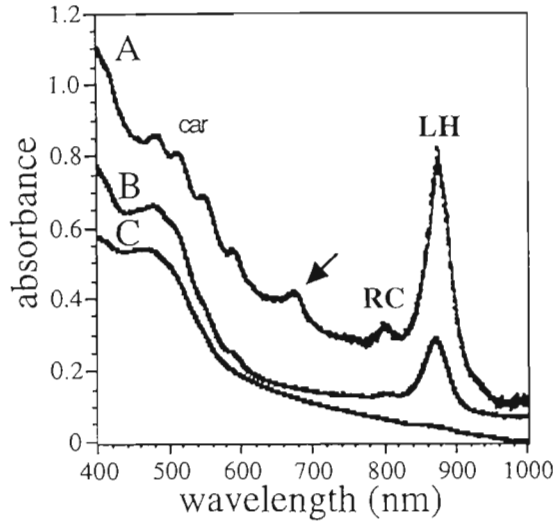

Fig. 3. Visible and near-infrared absorption spectra of membranes of ORS278 and 278Δ*pufLM* strains. Line A corresponds to the spectrum of membranes of strain ORS278 isolated from stem nodules and line B corresponds to that of bacteria grown *ex planta*. Because of the high scattering properties of the bacteroid preparation, the optical density at 1000 nm has been arbitrary decreased from 1 to 0.1. Line C corresponds to the absorption of membranes isolated from strain 278Δ*pufLM*. The arrow indicates a small contamination by chloroplasts in the bacteroid preparation. Absorption of the RC, light-harvesting complexes (LH), and carotenoids (car) is also indicated.

The region upstream of the *pufB* gene of *Bradyrhizobium* sp. strain ORS278 contained a nucleotide sequence showing significant sequence identity (73%) with the 3′-region of *bchZ* of *R. rubrum*, which coded for an enzyme involved in the conversion of chlorophyll to bacteriochlorophyll (26). Downstream of the *pufM* gene, another ORF encoding a putative heme oxygenase was identified unexpectedly. The use of a *hmuO*-specific probe demonstrated that ORS278 possesses a unique copy of the *hmuO* gene (data not shown). The enzyme encoded by this gene is highly conserved throughout the animal and plant kingdoms. It catalyzes the cleavage of the heme ring with the production of biliverdin, Fe^{3+}, and carbon monoxide (27, 28). The putative HmuO protein contained 203 amino acids and showed homology with the corresponding genes from a variety of organisms: 36% with *Synechocystis*, 36% with the human HO-1, 35% with *Rhodella violacea*, and 30% with *Corynebacterium diphtheriae*. Taking into account conservative substitutions of amino acids, similarities reached 49%, 49%, 46%, and 45%, respectively.

The *pufM* and *hmuO* genes are closely linked physically—i.e., the supposed ATG start codon of *hmuO* was located 94 bp after the *pufM* stop codon. This could indicate that the *hmuO* gene belongs to the *puf* operon. However, a putative hairpin loop structure was detected between these two genes. This hairpin was characterized by a stem of 11 bp adjacent to 4 T residues, and indicating a possible transcriptional termination signal after the *pufM* gene. An additional putative hairpin loop structure (stem of 8 bp adjacent to 3 T residues) was identified downstream from the *hmuO* gene, suggesting that the *hmuO* gene corresponds to a single transcript.

Mapping of the *puf* and *hmuO* Start Sites. Primer extension analysis had a twofold objective: (*i*) to identify the precise 5′ end of the *puf* operon and (*ii*) to determine whether the *hmuO* gene belongs to another transcription unit. The ^{32}P-labeled primers (pufB.rev and hmuO.rev, see *Materials and Methods*) were hybridized to total RNAs isolated from cells grown under either continuous dark (D) or a dark–light cycle (P). For the *puf* operon start site analyses (Fig. 2*A*), only the dark–light cycle culture condition gave one single transcriptional initiation site, which corresponded to a T on the RNA level located 111 bp upstream from the putative translational start of *pufB*. Results obtained with the hmuO.rev primer showed the presence of one 5′ end of *hmuO* mRNA corresponding to a T located 153 bp upstream from the putative translational start of the *hmuO* gene (Fig. 2*B*).

Photosynthetic Activity in *Bradyrhizobium* Sp. Strain ORS278 and in *puf* and *hmuO* Mutants. Fig. 3 shows a comparison of the absorption spectra of membranes isolated from the wild-type strain grown under cyclic illumination *ex planta* and from membranes isolated from *A. sensitiva* stem nodule endophytes. Both types of membranes display absorption bands characteristic of the presence of light-harvesting complexes, RCs, and carotenoids. The near-infrared part of the spectrum is very similar to the one observed for *Rhodospirillum rubrum* membranes, demonstrating the absence of light-harvesting II complexes. The photosynthetic apparatus is expressed only by cells grown under a light–dark cycle (not shown), as has already been described for BTAi 1 (3).

The photosynthetic activity of the bacteria was determined by monitoring light-induced absorption changes in intact cells. Fig. 4*A* shows the kinetics of the light-induced changes observed in cells grown under a light–dark cycle and placed under aerobic conditions at 422 nm, a wavelength characteristic of the redox state of the cytochromes. Under these conditions, a biphasic photo-oxidation of a cytochrome is observed with a fast phase unresolved (<50 μs) and a slow phase with a halftime of 200 μs (not shown), followed by its reduction in the 10-ms time range. The light-induced difference spectra, detected 50 μs and 1 ms after the actinic flash, confirm the photo-oxidation of a cyto-

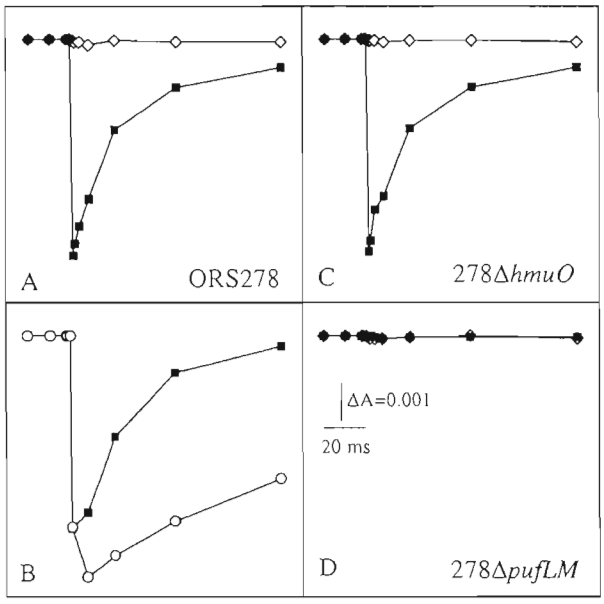

Fig. 4. The kinetics of cytochrome rereduction measured at 422 nm on whole cells of *Bradyrhizobium*. The cells were resuspended in fresh growth medium made either aerobic by bubbling air or anaerobic by bubbling argon through the suspensions. (*A*) Strain ORS278, aerobic (■) or anaerobic (◇) conditions. (*B*) Strain ORS278, aerobic condition in the absence (■) or presence (○) of 40 μM myxothiazol. (*C* and *D*) Strains 278Δ*hmuO* and 278Δ*pufLM*, aerobic (■) or anaerobic (◇) conditions.

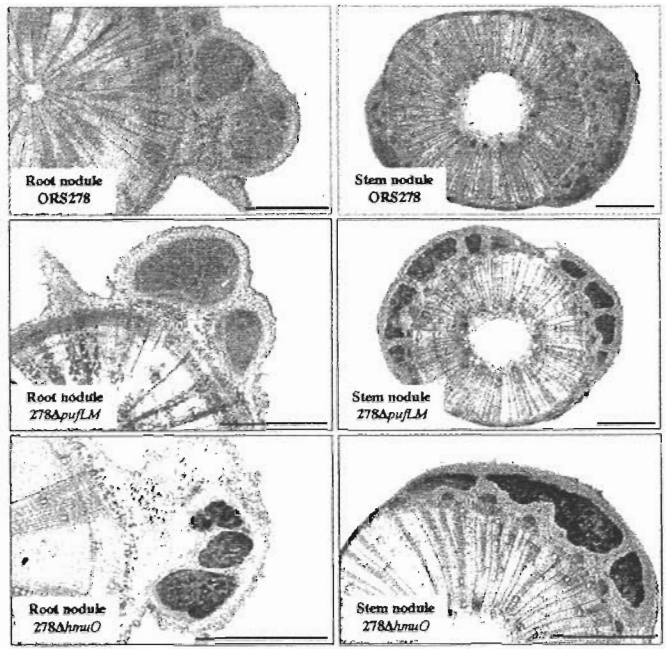

Fig. 5. Histochemical localization of β-galactosidase activity in stem and root nodules inoculated with ORS278, 278Δ*pufLM*, and 278Δ*hmuO* strains. (All magnification bars = 1 mm.)

chrome (not shown). The addition of myxothiazol, a specific inhibitor of the bc_1 complex, strongly slows the kinetics of rereduction of the photo-oxidized cytochrome (Fig. 4*B*). When the cells are placed under anaerobic conditions, no light-induced absorbance changes can be detected (Fig. 4*A*), in agreement with the aerobic character of the bacterium. Light-induced absorption changes have also been performed with intact cells to assay the photochemical competence of 278Δ*pufLM* and 278Δ*hmuO*, mutants deleted in the *pufLM* and *hmuO* genes, respectively (Fig. 1). As expected, no light-induced photo-oxidation of cytochrome can be detected upon illumination of 278Δ*pufLM* cells even under aerobic conditions (Fig. 4*D*). In addition, the deletion of the RC genes induces a profound modification of the absorption spectrum of the intracytoplasmic membrane (Fig. 3*C*). On the other hand, the photochemical activity and the absorption spectrum of the 278Δ*hmuO* mutant grown under cyclic illumination were found to be identical to the one observed for the wild-type strain (Fig. 4*C* and data not shown).

***In Planta* and *ex Planta* Expression of the *puf* and *hmuO* Genes.** The expression of the *puf* and *hmuO* genes was assayed *in planta* by inoculation of *A. sensitiva* with the two mutants bearing the *lacZ-hmuO* and *lacZ-puf* fusions. Nodules were harvested 20 days after inoculation, and transverse sections were stained for β-galactosidase activity (Fig. 5). A strong β-galactosidase activity was visualized in the central tissues of both stem and root nodules with the *lacZ-hmuO* fusion. In contrast, the *lacZ-puf* fusion shows a high β-galactosidase activity exclusively in the stem nodules. These results indicate that both *hmuO* and *puf* genes are expressed during symbiosis but that the *puf* genes are specifically expressed in the stem nodules, in agreement with the observation of the photosynthetic unit exclusively in these nodules (Fig. 3). *Ex planta*, we observed that *puf* expression requires a minimum of 3% oxygen (data not shown), confirming the aerobic character of *Bradyrhizobium* sp. strain ORS278.

Symbiotic Phenotypes of *puf* and *hmuO* Mutants. The two mutants, 278Δ*pufLM* and 278Δ*hmuO*, were used to investigate the requirement for bacterial photosynthesis and heme oxygenase on both root- and stem-nodulation, and nitrogen fixation during symbiosis. Root inoculation with both mutants, and stem inoculation with the 278Δ*hmuO* mutant, resulted in nodule number and plant dry weight similar to those inoculated with the wild-type strain (Fig. 6 and Table 1). The HmuO mutant induced stem nodulation and nitrogen fixation at a rate similar to that of the wild-type strain, suggesting that the HmuO activity is not an essential factor for symbiosis. In contrast, stem inoculation with the mutant 278Δ*pufLM* induced a lag of 3 days in nodulation (data not shown) and a drastic decrease (about 50%) in stem nodule number, plant growth, and nitrogenase activity (Table 1). These results, observed in three independent experiments, demonstrate that bacterial photosynthesis plays an important role in stem nodulation efficiency.

It is noteworthy that no difference is observed in the stem nodule structures induced by the wild-type or mutant 278Δ*pufLM* (Fig. 5). Furthermore, the few nodules formed by the photosynthetic mutant exhibited the same individual nitrogenase activity as the wild-type nodules (Table 1). In addition, we observed that the expression of *puf* genes is already detected 5 days after inoculation, before the appearance of the nodules, specifically at the emergence of root primordia (data not shown). These data indicate that bacterial photosynthesis plays a role in an early step of infection rather than in the energy requirement for nitrogenase activity.

Discussion

Absorption spectroscopy and photochemical measurements clearly indicated the presence of a functional photosynthetic apparatus in intact cells of ORS278. The occurrence of a light-induced cyclic electron transfer involving electron transfer between the RC and cytochromes bc_1 is attested by the fast photo-oxidation of a cytochrome *c* after its rapid rereduction, a reaction specifically inhibited by myxothiazol. These results show that a complete photoinduced electron transfer occurs in a

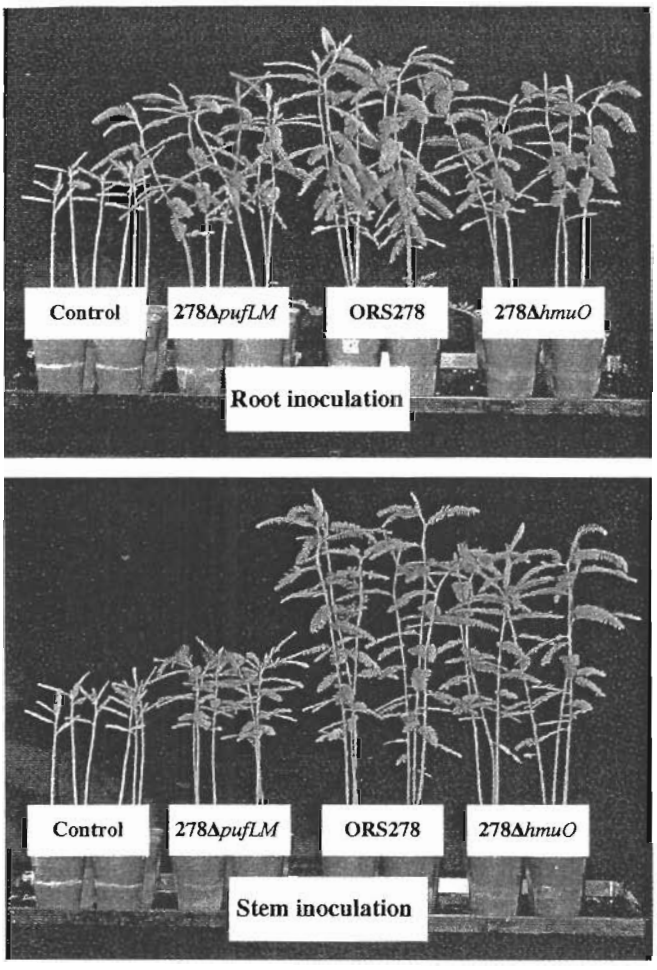

Fig. 6. Comparison of the growth of the plants of *A. sensitiva*, roots or stems inoculated with ORS278, 278Δ*pufLM*, and 278Δ*hmuO* strains. Photographs were taken 5 weeks after inoculation.

photosynthetic *Bradyrhizobium*, in full agreement with several studies reported for the BTAi 1 strain (3). While typical photosynthetic bacteria can grow under continuous light and anaerobiosis, the synthesis of the photosynthetic apparatus of ORS278 occurs only under cyclic illumination, and its photosynthetic activity requires aerobic conditions. The inability of aerobic photosynthetic bacteria to grow at the expense of light under anaerobic conditions has been related to the reduction of the primary electron acceptor because of the more positive value of its midpoint potential compared with those of the purple photosynthetic bacteria (29–31). However, in a recent report, Scharze *et al.* (32) cast doubt on this interpretation, since they observed that the high value for the midpoint potential of the primary electron acceptor obtained for *Roseobacter denitrificans* (29) was due to lack of equilibration during the redox titration.

The organization of the *puf* operon of strain ORS278 is one of the simplest described so far because it contains only the *pufB*, *pufA*, *pufL*, and *pufM* genes. However, the region downstream of the *puf* operon surprisingly shows a putative ORF encoding a heme oxygenase. Start point analysis and *lacZ* fusion studies (data not shown) showed that the expression of the *hmuO* gene is controlled by its own promoter located 153 bp in front of the putative start codon. Nevertheless, we cannot completely exclude that the *hmuO* gene could also be partially transcribed from the *puf* operon. Two main functions of heme oxygenase have been proposed for the bacteria in which this gene has been identified recently. In *Corynebacterium diphtheriae*, HmuO is involved in the release of iron from hemoglobin (33), whereas in the cyanobacterium *Synechocystis* this enzyme is involved in the biosynthesis of linear tetrapyrroles that act as chromophores in phycobilins (34). We propose instead that the *hmuO* gene is involved, as in both cyanobacteria and plants, in the chromophore biosynthesis of a regulatory receptor. Our proposal is based on the localization of this gene near the photosynthetic gene cluster and the assumptions that light exerts its regulatory influence on bacteriochlorophyll accumulation in BTAi 1 via photoreceptors (3, 5).

Phylogenetic analysis of *pufL* and *pufM* sequences available from photosynthetic α-proteobacteria (data not shown) shows that the *puf* genes of strain ORS278 are closely related to those of the anaerobic photosynthetic bacterium *Rhodopseudomonas palustris* (supported by a bootstrap value of 99%). Despite its functional similarity to aerobic photosynthetic bacteria, ORS278 is phylogenetically distant from *Roseobacter denitrificans* and *Erythrobacter longus*. A similar conclusion has also been deduced previously on the basis of a 16S rDNA analysis (35). The congruence of these two approaches indicates that the photosynthetic character of strain ORS278 is not the result of a lateral transfer, as suggested for other photosynthetic bacteria (36), but rather evolved from a photosynthetic ancestor common to *R. palustris*. Most bradyrhizobia are root symbionts living in an environment devoid of light. As a consequence, the photosynthetic system is no longer necessary and could have been progressively lost. In contrast, the maintenance of the photosynthetic function in the stem symbionts of *Aeschynomene* might

Table 1. Symbiotic phenotypes of 278Δ*pufLM* and 278Δ*hmuO* mutants compared with those of the wild-type strain ORS278

	Root inoculation			Stem inoculation			
Strain	No. of root nodules*/plant	Plant dry wt,† g/plant	Fixation activity,‡ μmol/h/plant	No. of stem nodules*/plant	Plant dry wt,† g/plant	Fixation activity,‡ μmol/h/plant	Fixation activity/nodule, μmol/h/nodule§
ORS278	33 ± 7	1.78 ± 0.3	63 ± 12	19 ± 3	1.94 ± 0.3	75 ± 11	4 ± 0.8
278Δ*pufLM*	29 ± 9	1.47 ± 0.2	57 ± 10	9 ± 3	0.97 ± 0.2	34 ± 5	3.8 ± 0.7
278Δ*hmuO*	32 ± 9	1.44 ± 0.3	51 ± 10	20 ± 4	1.62 ± 0.2	63 ± 11	3.3 ± 0.7
No inoculum	0	0.47 ± 0.1	0	0	0.47 ± 0.1	0	—

All values are the means ± standard errors of 10 individual plants.
*The number of nodules was measured 15 days after inoculation.
†The dry weight was measured 5 weeks after inoculation.
‡The acetylene reduction assay was performed 5 weeks after inoculation.
§These values were obtained by dividing the values of the acetylene reduction activity of the entire plant by the number of nodules measured on the stem 15 days after inoculation.

be the result of a selective advantage. This last hypothesis is in full agreement with our demonstration that the disruption of the photosynthetic genes induced a 50% decrease in stem-nodule number with the photosynthesis-negative mutant compared with either the wild-type strain or the HmuO mutant used as controls. This reduction of stem nodulation led to a decrease in the nitrogen fixation activity, and thus, plant growth. This result clearly demonstrates that bacterial photosynthesis plays a significant role in the efficiency of stem infection. In the stem-nodulating legumes, rhizobial infection starts through "crack entry"—e.g., bacteria gain access to the cortical cells of the host plant via ruptures in the stem epidermis caused by the emergence of root primordia (37, 38). *Sesbania rostrata,* nodulated by the nonphotosynthetic *Azorhizobium caulinodans* (1), and *Aeschynomene afraspera*, nodulated by both photosynthetic and nonphotosynthetic bacteria (13), are characterized by the presence on their stems of numerous protruding root primordia. These primordia pierce the epidermal layer and form at their base a large annular cavity (37, 38) where the bacteria can easily multiply. Therefore, the bacteria are protected from the outside environment and gain energy substrates from either cell debris or exudates. In contrast, in *Aeschynomene sensitiva*, most root primordia remain embedded beneath a few layers of epidermal cells and are rarely accessible to the bacteria (39). We suggest that, on the surface of the stem, under aerobic alternative light and dark conditions, the photosynthetic electron transfer chain contributes to an increase in the proton gradient for either ATP production or substrate transport. This energy either might allow the bacteria to survive on the stem where the availability of assimilable substrates is limited or be used by the bacteria to reach the cortical cells.

The high expression of the *puf* genes observed in the stem nodules and the detection of a functional photosynthetic unit in bacteroids indicate that the bacteria are photosynthetically active during stem symbiosis, as previously suggested (3, 4). We cannot exclude that this bacterial photosynthesis also plays an auxiliary role during the symbiosis by directly furnishing additional energy to the bacteroid. This might limit the demand for plant photosynthates and promote plant growth. The paradox of this last proposal is that the bacterial photosynthetic activity depends on aerobic conditions, whereas nitrogen fixation occurs only under microaerobic conditions. This implicates a fine regulation of the oxygen tension in the bacteroid, probably by means of leghemoglobin, to a narrow range where bacterial photosynthesis and nitrogenase activity can operate simultaneously.

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ARTICLE 15

Chaintreuil C, Boivin C, Dreyfus B, **Giraud E.**

Characterization of the common nodulation genes of the photosynthetic *Bradyrhizobium* sp. ORS285 reveals the presence of a new Insertion Sequence upstream of nodA

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Characterization of the common nodulation genes of the photosynthetic *Bradyrhizobium* sp. ORS285 reveals the presence of a new insertion sequence upstream of *nodA*

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Abstract

We isolated and characterized *nodA* genes from photosynthetic and non-photosynthetic rhizobia nodulating the legume genus *Aeschynomene*, and found that the *nodA* sequence from photosynthetic stem-nodulating bacteria was phylogenetically distant from the other already described *nodA* genes. Characterization of the photosynthetic strain ORS285 common *nod* gene cluster (*nodABC*) showed, upstream of *nodA*, the presence of a new insertion sequence element belonging to the IS3 family and specific to a group of photosynthetic strains from *Aeschynomene*.

Keywords: Nodulation gene; Photosynthetic *Bradyrhizobium*; nodA; Insertion sequence; *Aeschynomene*

1. Introduction

Rhizobia form nitrogen-fixing nodules on the roots of their leguminous host plants. Some species of the genus *Aeschynomene* have the unusual capacity to develop stem nodules, a property shared only with a very few species of the genera *Sesbania*, *Neptunia* and *Discolobium*. A number of stem isolates from *Aeschynomene* are of special interest because of their ability to develop a photosynthetic system [1], which is a rare trait in rhizobia. Three cross-inoculation groups among *Aeschynomene* species have been described [2]. Group I never forms stem nodules and is nodulated by the typical, broad host range, non-photosynthetic *Bradyrhizobium* strains. The two other groups, II and III, develop stem nodulation. Group III is exclusively nodulated by photosynthetic rhizobia, which are highly specific to that group [3]. Group II is intermediary as it is nodulated by both non-photosynthetic *Bradyrhizobium*, and with photosynthetic strains which are also able to nodulate Group III [3].

In the rhizobium–legume interaction, specificity is mainly controlled by Nod factors recognized by the host plant. All Nod factors are chitin oligomers mono *N*-acylated at the non-reducing end and diversely substituted at both ends of the molecules. These various substitutions, which confer plant-specificity, are encoded by host-specific *nod* genes. The synthesis of the *N*-acylated oligosaccharide core of the Nod factors is controlled by the *nodABC* genes, which are present in all rhizobia. The *nodA* gene, involved in the transfer of an acyl chain to the chitin oligosaccharide backbone Nod factor, has been shown to be a good nodulation marker [4].

In this study, we isolated and characterized the *nodA* genes of different photosynthetic and non-photosynthetic *Aeschynomene* rhizobia, and phylogenetically compared their sequence divergence. We found that the *nodA* gene sequence from photosynthetic bacteria was distant from that of the already described rhizobia and, therefore, we sequenced a 6.5-kb fragment containing the common nodulation gene *nodABC*. Here, we report the presence of a new insertion sequence element (IS) upstream of the *nodA* gene specific to Group II photosynthetic strains.

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2. Materials and methods

2.1. Bacterial strains

All the strains used are reported in Table 1. The strains were cultured on YEM medium [5] at 37°C. All strains were stored at −80°C on YEM medium adjusted to 20% (v/v) glycerol.

2.2. Polymerase chain reaction (PCR) amplification, DNA sequencing and sequence analysis

The *nodA* genes from Group I and II non-photosynthetic *Bradyrhizobium* strains were amplified using the degenerated primers nodAf.brad (5'-GTYCAGTGGAGSSTKCGCTGGG-3')/nodAr.brad (5'-TCACARCTCKGGCCCGTTCCG-3') whereas the *nodA* genes from the photosynthetic strains were amplified using the degenerated primers nodA1f (5'-TGCRGTGGAARNTRBVYTGGGAAA-3')/nodAb1r (5'GGNCCGTCRTCRAASGTCARGTA-3'). These four degenerate primers were defined from conserved motifs of NodA sequences available from the databanks. The primers Isf (5'-AGCCCAGCGACCTATTTTAG-3') and Isr (5'- GCCTCGATGAAAGCATTGTC-3') which amplified a 822-bp fragment inside the IS identified in ORS285 were used to check for the presence of this IS element in the different *Aeschynomene* symbionts.

A touchdown PCR was performed for primer pairs nodAf.brad/nodAr.brad and nodA1f/nodAb1r (annealing temperature 60 to 50°C in 20 cycles) as previously described [6]. For the primer pair Isf/Isr (annealing temperature of 55°C), the standard PCR method was used. PCR products were purified with a QIAquick Gel extraction Kit (Qiagen, France). The ABI Prism BigDye Terminator Cycle sequence Kit (Applied Biosystems, Foster City, CA, USA) was used to directly sequence the purified PCR with the primers used for the amplification. Sequencing reactions were analyzed on an Applied Biosystem model 310 DNA sequencer. An alignment was performed, using the program PILEUP [7], with a set of available NodA sequences. A phylogenetic tree was constructed by the neighbor-joining method [8], and a bootstrap confidence analysis was performed on 1000 replicates to determine the reliability of the tree topology so obtained [9].

2.3. Construction and screening of a genomic library of Bradyrhizobium ORS285

Total DNA of *Bradyrhizobium* strain ORS285 was subjected to a partial digestion with *Sau*3AI and dephosphorylated by alkaline phosphatase treatment. Fragments were then ligated to SuperCos I *Xba*I/*Bam*HI arms (Stratagene, La Jolla, CA, USA) as instructed by the manufacturer. Ligated DNA was packaged by using the Gigapack III Gold packaging extract (Stratagene). Screening was performed by PCR using the primers nodA.sp.285.f (5'-ACGCGCTCCCGTTCATGTCG-3')/nodA.sp.285r (5'-GAAATACAAGCACCAACGGC-3') which were designed from the *nodA* sequence of ORS285.

2.4. Nucleotide sequence accession number

The DNA sequences of the common *nod* genes of ORS285 strain have been submitted to the GenBank database under accession number AF284858.

3. Results and discussion

The *nodA* genes of both photosynthetic and non-photosynthetic *Aeschynomene* nodulating rhizobia were first isolated by PCR. By using the degenerated primers nodAf.brad/nodAr.brad, products of the expected size were obtained from all tested non-photosynthetic *Bradyrhizobium* strains isolated from either Group I (e.g. ORS301, ORS302, ORS304, ORS309) or Group II (e.g. ORS336). In contrast, all photosynthetic strains nodulating *Aeschynomene* Group II (e.g. ORS285, ORS287, ORS364) gave a positive amplification signal when using the degenerate primers nodA1f/nodAb1r. In spite of testing numerous primers defined from the consensus region of either NodA, NodB or NodC, we did not succeed in isolating the common *nod* genes from Group III photosynthetic strains. These results indicate a great divergence of the symbiotic genes as compared with the other rhizo-

Table 1
Characteristics of the strains used in this work

Bacterial strain	Original host plant	Group	Photosynthetic character	Reference
ORS301	*Agave americana*	I	−	[2]
ORS302	*A. pfundii*	I	−	[2]
ORS304	*A. elaphroxylon*	I	−	[2]
ORS309	*A. uniflora*	I	−	[3]
ORS285	*A. afraspera*	II	+	[3]
ORS287	*A. afraspera*	II	+	[3]
ORS336	*A. afraspera*	II	−	[3]
ORS364	*Acacia nilotica*	II	+	[3]

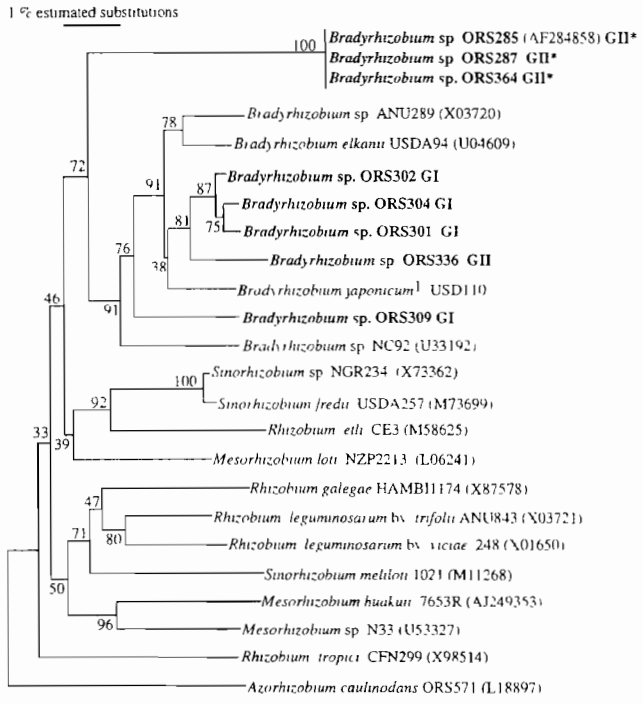

Fig. 1. Phylogenetic tree based on the NodA sequences using the neighbor-joining method. Bootstrap values, expressed as percentages of 1000 replications are given at the branching points. Numbers in parentheses, are the accession numbers of the sequences used. The NodA sequence of the *Bradyrhizobia* belonging to Group I and II (presented in bold) are available upon request. The NodA sequence from *B. japonicum* USDA110 was kindly provided by Tomasz Stepkowski. The bar represents one estimated substitution per 100 nucleotide positions. *, photosynthetic strain. GI, Group I. GII, Group II.

bia, which could be related to the extreme narrow host-specificity of the Group III photosynthetic strains.

Sequence analysis revealed that the *nodA* gene sequences from Groups I and II non-photosynthetic *Bradyrhizobium*-tested strains are polymorphs with a percentage of similarity ranging from 73.78 to 96.62% at the DNA level. In contrast, the *nodA* gene sequences of the Group II photosynthetic strains were found to be 100% identical, demonstrating that this symbiotic gene is very well conserved between these photosynthetic *Bradyrhizobium*. Phylogenetic NodA sequence analysis between the different rhizobia revealed that the *nodA* gene from the Group II photosynthetic strains constituted a new branch, related but distinct from the other *Bradyrhizobium nodA* gene (Fig. 1). Sequence similarity of NodA from those photosynthetic strains with other NodA *Bradyrhizobium* species was low (63%). These data are in full agreement with previous phylogenetic studies, which showed that photosynthetic *Aeschynomene* strains form a homogeneous and distinct cluster [3], for which a new species should be proposed. Conversely, NodA sequences from the non-photosynthetic Group I and II strains intertwined with the other species of *Bradyrhizobium* (Fig. 1), and the sequence similarity for this genus was high, ranging from 80 to 88%, thus confirming their taxonomic position within the *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* branches [3].

The high level of divergence of the NodA sequence of photosynthetic strains led us to characterize their complete common *nodABC* genes. Two specific primers nodA.sp 285.f/nodA.sp.285r based on *nodA* gene sequence were designed for the PCR screening of a DNA genomic library for strain ORS285 used as a model for Group II photosynthetic *Bradyrhizobium*. A positive clone (pSTM75) which contained an insert of approximately 35 kb was used to characterize the common nodulation genes.

A 6.5-kb region in the inserted DNA fragment of the pSTM75 cosmid, showing a positive hybridization signal to the *nodA* gene used as probe, was sequenced and analyzed, as shown in Fig. 2. This nucleotide sequence had four open reading frames (ORFs) encoding proteins with similarity to known Nod proteins (Fig. 2). Based on sequence similarities, three of these ORFs located from position 3992 to 6407 were assigned to *nodA*, *nodB* and *nodC* genes which presented the same classical organization as already found for other rhizobia. The *nodB* sequence showed 62 to 61% homology with the other *Bradyrhizobium nodB* genes available in databanks, and the *nodC* sequence, 63% homology with that of *Rhizobium* sp. strain N33 and 53% homology with the *nodC* gene of *Bradyrhizobium* sp. strain SNU001. The fourth ORF encoding a putative Nod protein was located from position 1746 to 617 and showed 55% amino acid homology with the *nolL* gene from *Rhizobium etli* (Fig. 2). The *nolL* gene has been shown to be responsible for the acetylation of the fucosyl residue in the nodulation factor [10], which indicates that Nod factors of photosynthetic strains are fucosylated, as already found for all *Bradyrhizobium* studied so far [11–12]. Indeed, we have detected by PCR in strain ORS285 (data not shown) a sequence homologous to the *nodZ* gene which encodes a fucosyl transferase.

Interestingly, we did not find a *nodD* regulatory gene upstream of *nodA*, as usually described for most rhizobia. Instead, we found in the 2-kb region located between *nodA* and *nolL*, an unexpected putative insertion element showing homology to IS sequences belonging to the IS3 family [13]. The analysis of this IS sequence revealed the presence of two ORFs, *ORFA* and *ORFB* (Fig. 2). The *ORFA*, starting with an ATG, encoded a putative protein of 88

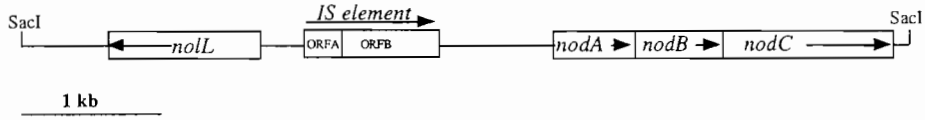

Fig. 2. Physical map of the common nodulation gene region in *Bradyrhizobium* sp. strain ORS285.

amino acids and showed a strong homology to a putative transposase identified in *Yersinia pestis* (59% of identity), the *ORFA* of IS*D*, an insertion element found in *Desulfovibrio vulgaris* (50% of identity) [14], the *ORFA* of IS1222 from *Enterobacter agglomerans* (48% of identity) [15], and also other members of the IS3 family. The *ORFB*, for which the proposed start codon overlapped the stop codon of *ORFA*, encoded a putative protein of 244 amino acids and also showed homology to the same ISs of the IS3 family. The greatest homologies found were 41% identity with the *Yersinia pestis* putative transposase, and 38 and 32% identity with the *ORFBs* of IS1222 [14] and IS*D1* [15], respectively.

The distribution of this IS element among *Aeschynomene* rhizobia was investigated by PCR. All of the photosynthetic strains from Group II, and only those, gave an amplified fragment of the expected size, indicating that this IS element is specific to this group of photosynthetic *Bradyrhizobium*. IS elements have been identified as mobile DNA elements in the genome of a wide range of bacterial genera and species [13]. They have been postulated to play an important role in the evolution and adaptation of bacteria. They have also been identified inside or flanking the symbiotic gene [16–18] where they could play an important role in the generation of nodulation polymorphism and consequently in the evolution of different symbiotic phenotypes [19]. Therefore, we can hypothesize that the transposition events within nodulation genes could have played a role, either by gene rearrangement or gene transfer, in the adaptation of photosynthetic bacteria to *Aeschynomene* stem-nodulation.

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ARTICLE 16

Sy A, **Giraud E**, Jourand P, Willem A, de Lajudie P, Samba R, Prin Y, Neyra M, Gillis M, Boivin C, Dreyfus B.

Methylotrophic *Methylobacterium* nodulate and fix nitrogen in symbiosis with legumes.

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Methylotrophic *Methylobacterium* Bacteria Nodulate and Fix Nitrogen in Symbiosis with Legumes

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Rhizobia described so far belong to three distinct phylogenetic branches within the α-2 subclass of *Proteobacteria*. Here we report the discovery of a fourth rhizobial branch involving bacteria of the *Methylobacterium* genus. Rhizobia isolated from *Crotalaria* legumes were assigned to a new species, "*Methylobacterium nodulans*," within the *Methylobacterium* genus on the basis of 16S ribosomal DNA analyses. We demonstrated that these rhizobia facultatively grow on methanol, which is a characteristic of *Methylobacterium* spp. but a unique feature among rhizobia. Genes encoding two key enzymes of methylotrophy and nodulation, the *mxaF* gene, encoding the α subunit of the methanol dehydrogenase, and the *nodA* gene, encoding an acyltransferase involved in Nod factor biosynthesis, were sequenced for the type strain, ORS2060. Plant tests and *nodA* amplification assays showed that "*M. nodulans*" is the only nodulating *Methylobacterium* sp. identified so far. Phylogenetic sequence analysis showed that "*M. nodulans*" NodA is closely related to *Bradyrhizobium* NodA, suggesting that this gene was acquired by horizontal gene transfer.

Symbioses between leguminous plants and soil bacteria commonly referred to as rhizobia are of considerable environmental and agricultural importance since they are responsible for most of the atmospheric nitrogen fixed on land. Rhizobia are able to elicit on most of the 18,000 species of the *Leguminosae* family the formation of specialized organs, called nodules, in which they reduce atmospheric nitrogen to ammonia to the benefit of the plant. Nodule formation is controlled by extracellular bacterial signal molecules, called Nod factors, which are recognized by the host plant (21, 34). The rhizobial species described so far are very diverse and do not form an evolutionary homogenous clade. They belong to three distinct branches within the α-2 subclass of *Proteobacteria* and are phylogenetically intertwined with non-symbiotic bacteria (40) (Fig. 1). A first large branch groups the genera *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, and *Allorhizobium* with *Agrobacterium*, a pathogenic bacterium of plants. A second branch contains the genus *Bradyrhizobium* together with photosynthetic free-living *Rhodopseudomonas*, whereas the third branch includes the genus *Azorhizobium* as well as the chemiautotroph *Xanthobacter*. Each rhizobial species has a defined host range, varying from very narrow, as in the case of *Azorhizobium caulinodans* (6), to very broad, as in the case of *Sinorhizobium* sp. strain NGR234 (30). Symbionts of legumes exhibiting ecological and agronomic potential should be characterized prior to their use in sustainable agriculture and environment management.

Crotalaria spp. are herbs and shrubs of the subfamily Papilionoideae; it is the largest plant genus in Africa. More than 500 species commonly occur in diverse climatological situations, from semidesert to rain forests and high mountains (1, 29). Some *Crotalaria* spp. are of great agronomic interest since they are used as green manure to improve soil fertility or control nematode populations in infested soils (4, 20). Characterization of a collection of rhizobia isolated from various *Crotalaria* species revealed two very distinct groups of symbiotic bacteria, a group of broad-host-range rhizobia related to *Bradyrhizobium* and a group of highly specific rhizobia of unknown taxonomic status (33).

We now report that the latter group of highly specific *Crotalaria* rhizobia belong to the *Methylobacterium* genus and assign them to a new species, for which we propose the name "*M. nodulans*." These *Methylobacterium* strains thus constitute a novel and fourth group of nitrogen-fixing legume-symbiotic bacteria. We demonstrated that "*M. nodulans*" is a facultative methylotroph, which is a unique property among rhizobia.

MATERIALS AND METHODS

Bacterial strains. Strains isolated from *Crotalaria* spp. are listed in Table 1. *Methylobacterium rhodesianum* LMG6086, *Methylobacterium organophilum* LMG6083, *Methylobacterium extorquens* LMG4250, *Methylobacterium rhodinum* LMG2275, *Methylobacterium zatmanii* LMG6087, *Methylobacterium mesophilicum* LMG5275, and *Methylobacterium* sp. strains LMG6378, LMG6085, and LMG6380 were from the collection of the Universiteit Gent (5). *Sinorhizobium meliloti* RCR2011, *Sinorhizobium medicae* A321, *Sinorhizobium fredii* USDA205, *Sinorhizobium terangae* ORS1009, *Rhizobium leguminosarum* bv. viciae 248, *Rhizobium etli* CFN42, *Rhizobium tropici* CIAT899, *Methylobacterium cicero* UPMCa-7, *Methylobacterium loti* NZP2213, *Bradyrhizobium japonicum* USDA110, *Bradyrhizobium elkanii* USDA61, *Allorhizobium undicola* ORS995, and *Azorhizobium caulinodans* ORS571 were from our collection. The growth medium for *Methylobacterium* strains, including "*M. nodulans*" ORS2060 and ORS1917, was M72 (5) supplemented with 50 mM methanol. The complete medium for other strains was YM (37). Nodulating *Methylobacterium* strains were grown at 37°C, other strains were grown at 30°C.

DNA technology. Genomic DNA was prepared by using the method of Chen and Kuo (7). Plasmid DNA was isolated with a Miniprep kit (Promega, Charbonières, France). PCR products were purified with a QIAquick gel extraction kit (Qiagen, Courtaboeuf, France). Restriction endonucleases and ligase were

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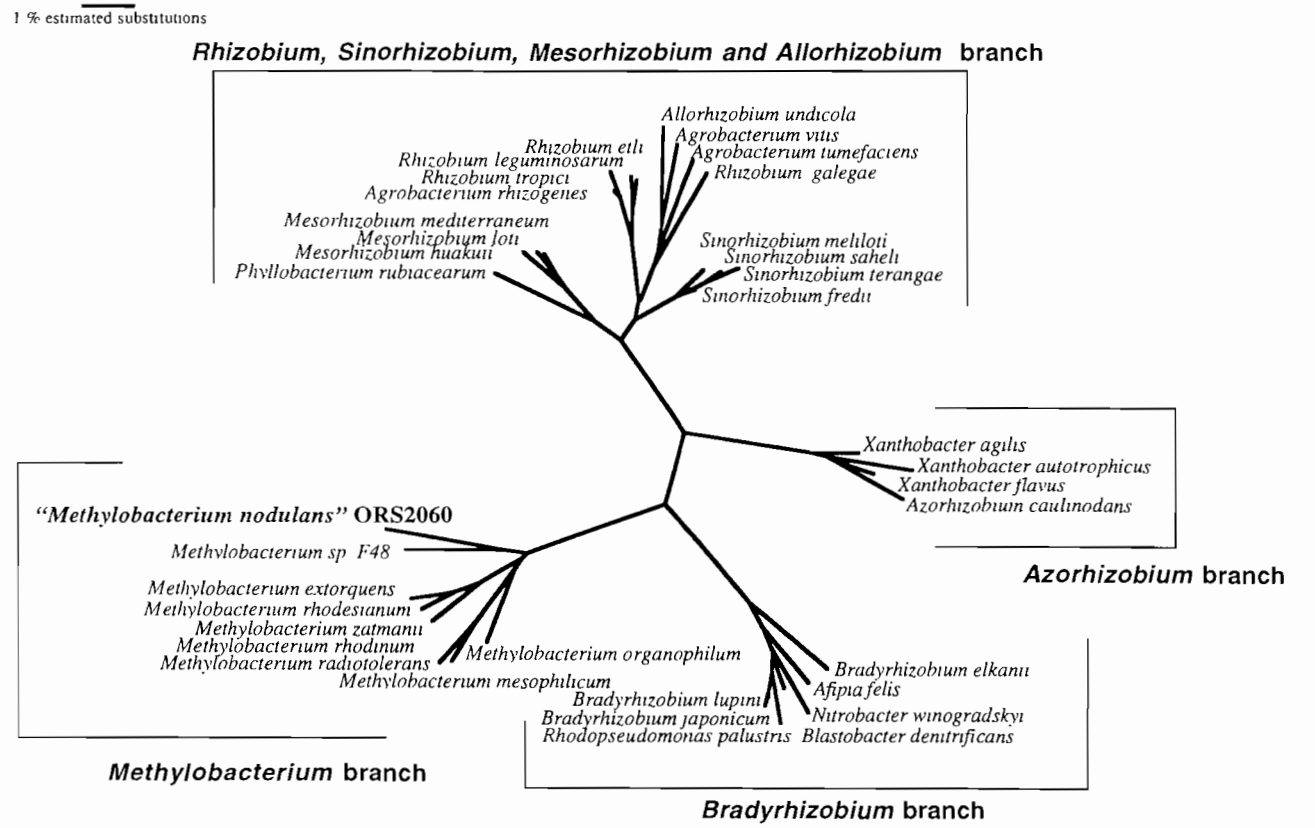

FIG. 1. Unrooted phylogenetic tree showing the different rhizobial branches, including the new "*M. nodulans*" in the α subdivision of *Proteobacteria*. The tree was constructed by using the neighbor-joining method from almost full-length 16S rDNA sequences. The GenBank/EMBL accession numbers are as follows (the first letters of the genus and species are given in parentheses): D 12790 (Pr), D12797 (Mh), X67229 (Ml), L38825 (Mmed), X67224 (Ar), X67234 (Rt), U29386 (Rl), U28916 (Re), Y17047 (Au), X67225 (Av), X67223 (At), X67226 (Rg), X67222 (Sm), X68390 (Ss), X68387 (St), X67231 (Sf), X94198 (Xag), X94201 (Xau), X94199 (Xf), D11342 (Ac), U35000 (Be), M65248 (Af), L11661 (Nw), S46917 (Bd), D25312 (Rp), D12781 (Bj), U69637 (Bl), D32226 (Mo), D32225 (Mmes), D32227 (Mrad), D32229 (Mrhodi), D32230 (Mz), D32228 (Mrhode), D32224 (Me), D32236 (Msp), and AF220763 (Mn).

used according to the manufacturer's specifications (Roche, Meylan, France, or Eurogentec, Seraing, Belgium). For Southern blot hybridization, restricted DNA was blotted to positively charged nylon membranes by the alkali transfer procedure and hybridized with digoxigenin (DIG)-dUTP using the DIG labeling kit supplied by Roche.

DNA amplification, sequencing, and analysis. The primers used for DNA amplification and sequencing are described in Table 2. Nearly full-length 16S ribosomal DNA (rDNA) was amplified using the universal eubacterial 16S rDNA primers FGPS6 and FGPS1509 (28). To perform 16S rDNA PCR-restriction fragment length polymorphism analysis, 1,500-bp PCR products were digested with *Sau*96I, *Hin*fI, *Msp*I, and *Hae*III and restriction fragments were analyzed by horizontal agarose gel electrophoresis using Metaphor agarose (FMC Bioproducts, Hellerup, Denmark). The 1,500-bp fragments of ORS2060 and ORS1924 were sequenced by using the primers FGPS6, FGPS1509, 16S-370f, 16-1080r, 16S-870f, and 16S-1924r. 555-bp sequences homologous to the *mxaF* gene were amplified from *Crotalaria* rhizobia and sequenced by using the nondegenerate primers f1003 and r1561 (26). For rhizobial species, a fragment of about 440 bp homologous to *mxaF* was amplified and sequenced by using the degenerate primers mxaf916 and mxar1360. Two pairs of primers, nodAfbrad/nodArbrad and nodA1f/nodAb1r, were tested for *nodA* amplification of reference *Methylobacterium* strains (LMG6086, LMG6083, LMG4250, LMG2275, LMG6087, LMG5275, LMG6378, LMG6085, and LMG6380). *nodA* amplification and sequencing of ORS2060 were performed using three pairs of primers, nodAfbrad/nodArbrad, nodboxuniv2/nodArbrad, and nodAfbrad/NodB76r.

PCR amplification was performed with a Perkin-Elmer model 2400 thermocycler in a 25-μl (total volume) reaction mixture containing 50 ng of genomic DNA, each deoxynucleotide triphosphate (200 μM), primers (0.8 μM each), $MgCl_2$ (1.5 mM), 1.25 U of *Taq* DNA polymerase (Gibco BRL, Cergy Pontoise, France), and the buffer supplied with the enzyme. A touchdown PCR (12) was performed for primer pairs nodAfbrad/nodArbrad and nodA1f/nodAb1r (annealing temperature from 65 to 55°C in 20 cycles), primer pairs nodboxuniv2/nodArbrad and nodAfbrad/NodB76r (annealing temperature from 60 to 45°C in 30 cycles), and mxaf916/mxar1360 (annealing temperature from 60 to 50°C in 20 cycles). A standard PCR method was used for primer pairs FGPS6/FGPS1509 (60°C annealing temperature) and f1003/r1561 (55°C annealing temperature). Sequencing reactions were performed with the ABI Prism BigDye Terminator Cycle sequence kit (Applied Biosystems, Foster City, Calif.) and analyzed on an

TABLE 1. *Crotalaria* rhizobia used in this study

Strain(s)[a]	Host plant
"M. nodulans"	
ORS1917, ORS1991, ORS2060^T	*C. podocarpa*
ORS1924, ORS1928, ORS1937, ORS2030, ORS2092	*C. perrottetii*
ORS2026, ORS2045, ORS2076	*C. glaucoides*
Bradyrhizobium sp.	
ORS1810	*C. lathyroides*
ORS1816	*C. hyssopifolia*
ORS1813	*C. hyssopifolia*
ORS1929	*C. comosa*
ORS2077	*C. retusa*
ORS2088	*C. goreensis*

[a] All strains are described by Samba et al. (33).

TABLE 2 Primers used for DNA amplification and sequencing

Primer name	Primer sequence	Target gene	Reference
FGPS6	5′-GGA GAG TTA GAT CTT GGC TCA G-3′	16S rRNA	28
FGPS1509	5′-AAG GAG GGG ATC CAG CCG CA-3′	16S rRNA	28
16S-370f	5′-CCT GGG GAG TAC GGT CGC AAG-3′	16S rRNA	This study
16S1080r	5′-GGG ACT TAA CCC AAC ATC T-3′	16S rRNA	This study
16S-870f	5′-CCT GGG GAG TAC GGT CGC AAG-3′	16S rRNA	This study
16S-1924r	5′-GGC ACG AAG TTA GCC GGG GC-3′	16S rRNA	This study
f1003	5′-GCG GCA CCA ACT GGG GCT GGT-3	*mxaF*	26
r1561	5′-GGG CAG CAT GAA GGG CTC CC-3′	*mxaF*	26
mxaf916	5′-GGC GAC AAC AAG TGG WCS ATG-3′	*mxaF*/*xoxF*	This study
mxar1360	5′-ART CCA TRC ARA YGT GGT T-3′	*mxaF*/*xoxF*	This study
xoxFr	5′-CCG GAA CGG CTC GTA RTC CA-3′	*xoxF*	This study
nodAfbrad	5′-GTY GAG TGG AGS STK CGC TGG G-3′	*nodA*	This study
nodArbrad	5′-TCA CAR CTC KGG CCC GTT CGG-3′	*nodA*	This study
nodA1f	5′-TGC RGT GGA ARN TRB VYT GGG-3′	*nodA*	This study
nodAb1r	5′-GGN CCG TCR TCR AAS GTC ARG TA-3′	*nodA*	This study
nodboxuniv2	5′-ATC NAA ACA AWN RAT TTT AC-3′	*nod* box	This study
NodB76r	5′-GGR TKN GGN CCR TCR TCR AAN GT-3′	*nodB*	This study

Applied Biosystems model 310 DNA sequencer Sequences were aligned by using the PILEUP program (11) Phylogenetic trees were constructed by the neighbor-joining method (32), and a bootstrap confidence analysis was performed on 1,000 replicates to determine the reliability of the tree topology obtained (13)

Rhizobial *mxaF*-homologous partial sequences are available upon request.

Construction and screening of a genomic library of ORS2060. To obtain a genomic library of the ORS2060 strain, total DNA was subjected to partial digestion with *Sau*3AI and dephosphorylated with an alkaline phosphatase treatment Fragments were then ligated to SuperCos I *Xba*I/*Bam*HI arms (Stratagene, La Jolla, Calif) as instructed by the manufacturer Ligated DNA was packaged by using the Gigapack III Gold packaging extract (Stratagene) Standard methods were used for titrating and cosmid propagation using *Escherichia coli* XL1-MR as the host Approximately 2,000 white colonies were picked individually into 96-well microtiter plates containing Luria-Bertani medium plus kanamycin (50 μg/ml), grown overnight at 37°C, and stored with 30% glycerol at −80°C Screening of *mxaF*-containing cosmids was performed by DNA amplification using the primer pair f1003/r1561 A selected clone, pSTM217, was confirmed by hybridization with an *mxaF* probe constructed by DIG labeling the ORS2060 555-bp *mxaF* internal fragment

Methanol utilization tests. Cells were grown for 48 h to mid-log phase in minimum mineral medium M72 (5) supplemented with pyruvate (10 mM) and yeast extract (0 5g/liter) Bacterial suspensions were diluted in M72 medium to an optical density of 0 05, and one of the following compounds was added methanol (MeOH) (10, 50, 100, or 500 mM), pyruvate (10 mM), or succinate (10 mM). Growth was monitored by measuring optical density at 620 nm. MeOH dosage in culture supernatants was performed as described previously (39) except that $KMnO_4$ was replaced by alcohol oxidase (EC 1 1 3 13) at 0 1 U/ml (Sigma, L'Isle d'Abeau, France)

Plant tests. Plant cultivation and nodulation tests were carried out as described previously (23), with the following modifications, seeds were superficially sterilized with concentrated sulfuric acid for 35 min (*Crotalaria podocarpa*), 30 min (*Crotalaria perrottetii*), 15 min (*Crotalaria comosa*), 40 min (*C goreensis*), or 25 min (*Crotalaria ochroleuca*). Effectiveness was estimated by visual observation of plant vigor and foliage color of 30-day-old plants

Fresh nodules were observed under an Olympus SHZ 10 stereomicroscope Sections of 80-μm thickness were made using a Leica VT1000S Vibratome Microscopic preparations were examined without further staining with an Olympus Provis microscope.

Bacteriochlorophyll detection. The presence of bacteriochlorophyll *a* in "*M. nodulans*" ORS2060 was checked as described previously (22)

Nucleotide sequence accession numbers. Accession numbers for 16S rRNA and the *mxaF* and *nodA* genes are as follows AF220762 (ORS1924 16S rRNA gene), AF220763 (ORS2060 16S rRNA gene), AF220764 (ORS2060 *mxaF* gene), and AF266748 (ORS2060 *nodA* gene) Accession numbers for rhizobial partial *mxaF*-homologous sequences are AF304307 to AF304313.

RESULTS

Bacteria that specifically nodulate *Crotalaria* belong to the *Methylobacterium* genus. Rhizobia isolated from *Crotalaria glaucoides*, *C. perrottetii*, and *C. podocarpa* were previously shown to be highly specific, since they effectively nodulated only these three species (33). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis grouped almost all the strains into three related electrophoretic clusters separated from other rhizobial species (33).

To determine the bacterial genus to which they belong, 16S rDNA analysis was performed on 11 representative strains belonging to the previously identified sodium dodecyl sulfate-polyacrylamide gel electrophoresis clusters (Table 1). All strains tested gave identical patterns by 16S rDNA PCR-restriction fragment length polymorphism analysis, showing that the strains form a very homogenous group. The 16S rRNA genes of two strains, ORS2060 isolated from *C. podocarpa* and ORS1924 isolated from *C. perrottetii*, were sequenced and shown to be identical (accession numbers AF220763 and AF220762, respectively). Phylogenetic 16S rDNA sequence analysis revealed that this group was distinct from the three main branches containing all known rhizobial species. Surprisingly, the specific *Crotalaria* symbionts belonged to the *Methylobacterium* lineage of the α-*Proteobacteria*, thus constituting a fourth phylogenetic rhizobium branch (Fig. 1). Sequence similarities with the different *Methylobacterium* species described so far ranged from 93.64% (*Methylobacterium radiotolerans*) to 94.52% (*M. extorquens*) and was 95.16% with its closest phylogenetic neighbor, *Methylobacterium* sp. strain F48. These ranges are comparable with those found between most *Methylobacterium* species, thus demonstrating that the specific *Crotalaria* rhizobia phylogenetically belong to the *Methylobacterium* genus.

Bacteria of the new species "*M. nodulans*" are facultative aerobic methylotrophs. *Methylobacterium* spp. oxidase methanol via a key periplasmic enzyme, methanol dehydrogenase, which belongs to the family of the pyrroloquinoline quinone (PQQ)-linked enzymes that are known as quinoproteins (2, 3). Methanol dehydrogenase is an $\alpha_2\beta_2$ tetramer noncovalently

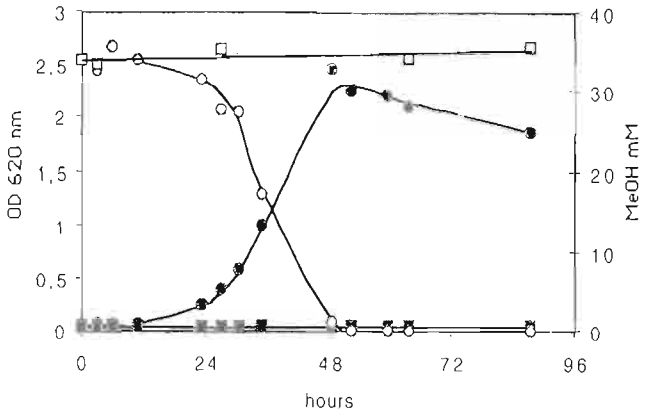

FIG. 2. Methanol utilization by "*M. nodulans*" ORS2060. *Bradyrhizobium* sp. strain ORS1810 (33) was used as a negative control. Shown is growth of ORS2060 (black circles) and ORS1810 (black squares) on minimum mineral medium 72 containing MeOH as the sole carbon source. MeOH concentrations in supernatants of cultures of ORS2060 (empty circles) and ORS1810 (empty squares) are also shown.

bound to PQQ (15). The structural gene for the α subunit of the methanol dehydrogenase encoded by *mxaF* is well conserved among gram-negative methylotrophic bacteria (24). Therefore, to evaluate the presence of methanol oxidation genes in *Crotalaria* rhizobia, we performed PCR amplifications using nondegenerate primers f1003 and r1561, defined from conserved parts of *mxaF* genes (26). These primers indeed produced an amplification product of the expected size for 9 of the 11 specific *Crotalaria* strains tested. The 555-bp PCR product obtained from the representative strain ORS2060 was homologous to *mxaF* genes. The full-length *mxaF* gene was obtained from a genomic library of ORS2060 probed with the 555-bp PCR product (see Materials and Methods for details). The corresponding nucleotide sequence contained a single extended 1,890-bp open reading frame (accession number AF220764) encoding a 629-amino-acid protein that exhibits 88% identity with the MxaF proteins of *M. extorquens* (accession number M31108) and *M. organophilum* (accession number M22629). A putative ribosome binding site was identified upstream from the proposed ATG start codon. The first 93 nucleotides of the structural gene encode a typical signal sequence for secretion (38). These results indicated that *Crotalaria*-nodulating *Methylobacterium* strains did contain, as all other *Methylobacterium* strains, the structural gene, *mxaF*, required for methanol oxidation.

In order to directly assess the methylotrophic properties of *Methylobacterium* species from *Crotalaria*, the strains were then tested for their ability to use methanol (50 mM) as the sole carbon source in liquid culture. Growth was compared to that of *M. extorquens* LMG4250 and *Bradyrhizobium* sp. isolated from *Crotalaria* species. No growth was observed for *Bradyrhizobium* strains over a 10-day period (Fig. 2). By contrast, all *Methylobacterium* strains from *Crotalaria* grew on this substrate, with generation times ranging from 9.5 to 40 h. The fastest growth rates were obtained with strains ORS2060 (Fig. 2) and ORS1917 (9.5-h doubling time) and were similar to the growth rate of *M. extorquens* LMG4250 on MeOH (50 mM) (7.5-h doubling time). Similar growth was noted on either pyruvate or succinate as the sole carbon source. Growth was obtained at an MeOH concentration up to 500 mM.

To confirm that the same bacterium isolated from *Crotalaria* exhibits both nodulation ability and methylotrophic properties, strain ORS2060 was grown on methanol, repurified from a single colony, and inoculated onto *C. podocarpa* and *C. perrottetii*. The bacteria formed nitrogen-fixing indeterminate nodules (Fig. 3). Single colonies reisolated from the nodules retained the ability to grow on methanol, clearly demonstrating that this new *Methylobacterium* strain is able both to grow on one-carbon (C_1) compounds and to effectively nodulate legumes.

Proposal of a new species was warranted by the low 16S rDNA sequence similarity values between *Methylobacterium* strains isolated from *Crotalaria* and other *Methylobacterium* strains. The unique symbiotic properties of these bacteria (see below) led us to propose the name "*Methylobacterium nodulans*." *Methylobacterium* spp., which are mostly isolated from water and leaf surface microflora, are known in the literature as pink-pigmented facultative methylotrophs (19). However, "*M. nodulans*" strains are not pigmented and the type strain,

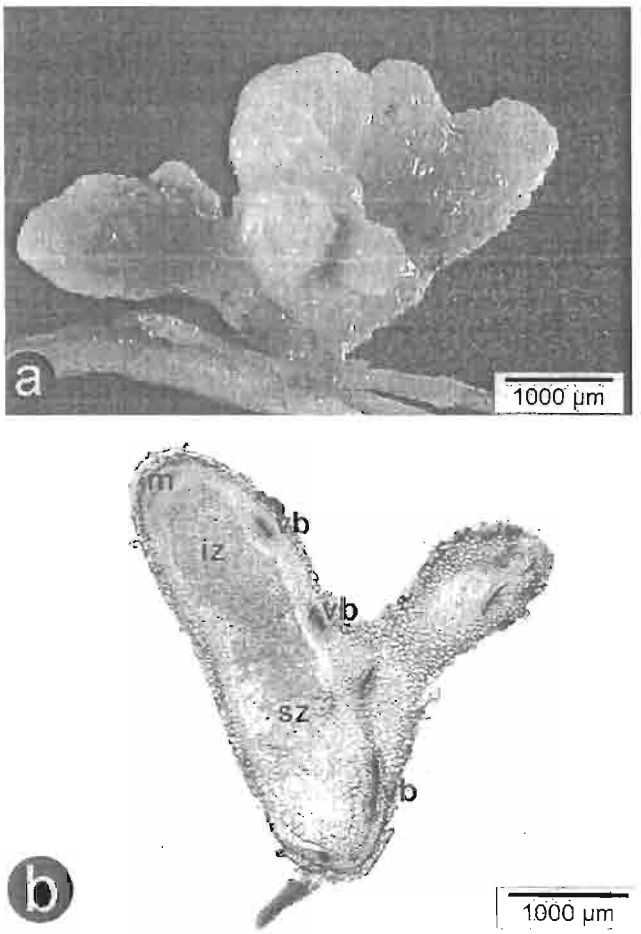

FIG. 3. Nodules of *C. perrottetii* inoculated with "*M. nodulans*" ORS2060. (a) Multilobed fresh nodule; (b) unstained longitudinal section displaying the classical structure of indeterminate nodules with an apical meristematic zone (m), an infection zone (iz), a senescent zone (sz), and peripheral vascular bundles (vb).

ORS2060, did not exhibit the characteristic bacteriochlorophyll absorption peak at 766 nm. This could account for their adaptation to the soil rather than to the phyllosphere or water.

"*M. nodulans*" is the only nodulating *Methylobacterium* species identified to date. The *Methylobacterium* genus has never been reported to contain nodulating bacteria. However, to be certain that nodulation is not a general but hitherto undetected *Methylobacterium* feature, we tested representative strains of several *Methylobacterium* species (*M. rhodesianum*, *M. organophilum*, *M. extorquens*, *M. rhodinum*, *M. mesophilicum*, *M. zatmanii*, *M. radiotolerans*, and "*M. nodulans*") and a few *Methylobacterium* sp. strains (LMG6378, LMG6085, and LMG6380) by inoculation on *Crotalaria* species nodulated by broad-host-range *Bradyrhizobium* (*C. comosa*, *C. goreensis*, and *C. ochroleuca*) or by "*M. nodulans*" (*C. perrottetii*). None of them nodulated, except "*M. nodulans*."

Specific rhizobial infection and nodulation of legumes is mainly controlled by a set of bacterial nodulation genes involved in the production of lipochitooligosaccharides (Nod factors) that act as morphogenic signal molecules on specific legume hosts (10, 36). Structural *nodABC* genes encoding key enzymes in Nod factor biosynthesis are present in all rhizobia. We thus looked for the presence of the *nodA* gene in the *Methylobacterium* representative strains listed above by PCR amplifications using two pairs of degenerate primers defined from conserved parts of NodA sequences. None of these strains responded positively, except "*M. nodulans*." The full-length *nodA* sequence of "*M. nodulans*" ORS2060 was determined after PCR amplification. The open reading frame, which probably corresponds to *nodA*, is 642 nucleotides long (accession number AF266748). Sequence similarity with the different complete rhizobial NodA protein sequences available in databases ranged from 53.06% (*A. caulinodans*) to 74.11% (*B. elkanii* USDA94). Phylogenetic analysis of available NodA proteins showed that "*M. nodulans*" is grouped with *Bradyrhizobium* spp. (Fig. 4).

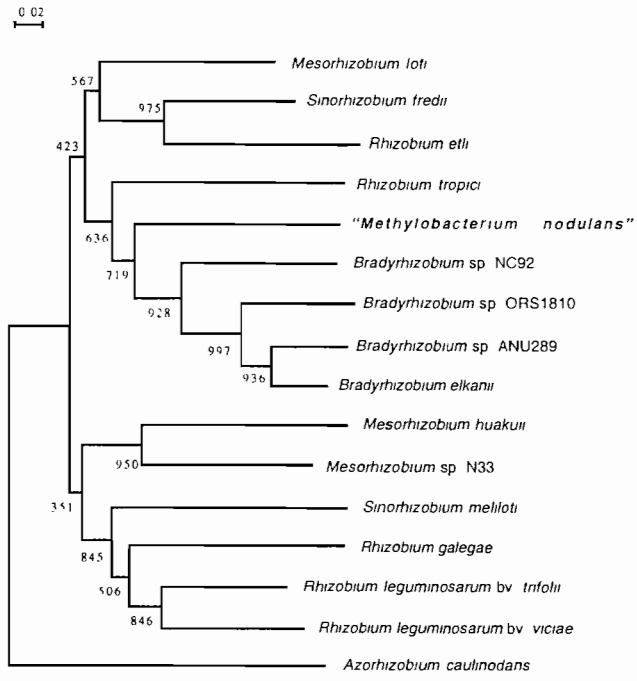

FIG. 4. Phylogenetic tree based on full-length NodA sequences construced by using the neighbor-joining method. Bootstrap values (from 1,000 replications) are indicated. The GenBank/EMBL accession numbers are as follows (the first letters of the genus and species are given in parentheses): L18897 (Ac), 106241 (Ml), M73699 (Sf), M58625 (Re), X98514 (Rt), AF266748 (Mn), U33192 (BspNC92), U04609 (BspANU289), U04609 (Be), AJ249353 (Mh), U53327 (MspN33), M11268 (Sm),X87578 (Rg), and X01650 (Rl). The NodA sequence of *Bradyrhizobium* sp. strain ORS1810 was kindly provided by T. Stepkowski.

Methylotrophy is not a common feature of rhizobia. We tested a collection of rhizobia belonging to various genera and species (*S. meliloti*, *S. medicae*, *S. fredii*, *S. terangae*, *R. leguminosarum* bv. viciae, *R. etli*, *R. tropici*, *M. ciceri*, *M. loti*, *B. japonicum*, *B. elkanii*, *A. undicola*, and *A. caulinodans*; see Materials and Methods) for their ability to grow on MeOH (50 mM) as the sole carbon source in solid and liquid culture. No growth of these strains was observed within 6 days. We also used PCR amplification to investigate the possible presence of a *mxaF*-homologous gene in the rhizobial strains. No amplification could be obtained using primers f1003 and r1561. When degenerate primers defined from conserved parts of both MxaF and MxaF homologs from α-*Proteobacteria* were used, all strains were found to contain a 440-bp sequence homologous to *mxaF*. These sequences however exhibited higher homology to two *mxaF* homologs, *mxaF'* from *M. extorquens* (72 to 80% amino acid identity) and *xoxF* from *Paracoccus denitrificans* (72 to 83% amino acid identity) than to *mxaF* from *M. extorquens* (50 to 53% amino acid identity). *xoxF* and *mxaF'* are thought to encode an alternative PQQ-dependent dehydrogenase (8, 18). Phylogenetic analysis further revealed that the rhizobial protein sequences are clustered with XoxF and MxaF' and separated from the methanol dehydrogenase MxaF from α- and β-*Proteobacteria* (Fig. 5). We thus conclude that the sequences found in rhizobia probably do not correspond to a bona fide *mxaF* ortholog.

DISCUSSION

The rhizobial species described so far belong to three distinct 16S rRNA branches within α-*Proteobacteria*, including nonsymbiotic bacteria such as the plant pathogen *Agrobacterium* and the human and animal pathogen *Afipia*. The discovery of a fourth rhizobial phylogenetic branch, constituted by bacteria of the *Methylobacterium* genus, thus confirms and extends the polyphyletic origin of rhizobia within the α subclass of *Proteobacteria* (40). It is noteworthy that although rhizobia have been studied for more than 100 years, symbionts of less than 50 of the 750 known legume genera have been fully characterized. Therefore, it is quite likely that much greater rhizobial diversity will be discovered by characterizing symbionts of unexplored legumes and by focusing on legumes of unexplored areas.

Bacterial nodulation (*nod*) genes have been shown to play a central role in the molecular dialog between the plant and the bacterium, leading to plant recognition, infection, and nodulation (10, 34, 36). The presence of structural *nodABC* genes in all rhizobia indicates the unique origin of these genes, which could have been disseminated among *Proteobacteria* via self-transmissible plasmids (25) or other mechanisms allowing

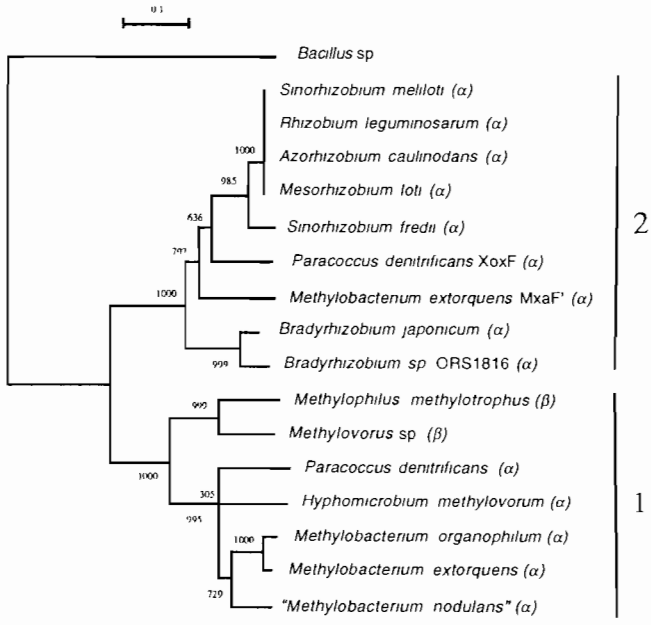

FIG. 5 Phylogenetic tree based on about 140-amino-acid MxaF-homologous sequences constructed by using the neighbor-joining method. Bootstrap values (from 1,000 replications) are indicated. Cluster 1 groups only MxaF sequences with the following GenBank/EMBL accession numbers (the first letters of the genus and species are given in parentheses): M17339 (Pd), AB004097 (Hm), M22629 (Mo), M31108 (Me), AF220764 (Mn), U41040 (Mm), and AF184915 (Msp). Cluster 2 groups the rhizobial sequences (accession numbers given in Materials and Methods) together with XoxF from *P. denitrificans* (U34346) and MxaF' from *M. extorquens* (U72662). MxaF from *Bacillus* sp. (M65004) was used as the root. The topology of the tree constructed with the full-length MxaF-homologous sequences (thus excluding the rhizobial sequences) is similar. (α), member of α-*Proteobacteria*; (β), member of β-*Proteobacteria*.

transfer of "symbiotic islands" (35). The presence of the *nodA* gene in "*M. nodulans*" is consistent with nodulation data and suggests that this bacterium is able to establish symbiosis by the same molecular mechanisms as other rhizobia. The NodA phylogenetic analysis confirms the monophyletic origin of this structural common *nod* gene (Fig. 4). The deduced absence of *nodA* in nonsymbiotic *Methylobacterium* spp. together with the close phylogenetic relationship between "*M. nodulans*" and *Bradyrhizobium* NodA proteins suggests that "*M. nodulans*" has acquired nodulation properties by lateral gene transfer.

Bacteria of the *Methylobacterium* genus are facultative methylotrophs capable of growing on one-carbon compounds such as formate, formaldehyde, and methanol as the sole source of carbon and energy, as well as on a wide range of multicarbon substrates (16, 17). They constitute one of the major methylotrophic branches in the α-2 subclass of *Proteobacteria*. It should be noted that other branches, including the three rhizobial branches described to date (Fig. 1), also contain methylotrophs. Indeed, *Xanthobacter* species are all autotrophic methylotrophs (27), most *Rhodopseudomonas* species grow photosynthetically with methanol (31), and a methyl bromide-utilizing methylotroph closely related to *Rhizobium* species was recently identified (9). *Methylobacterium* species are known to occur in man-made environments such as drinking water supplies, swimming pools, and hospital washbasins, where they often become highly resistant to chlorine. They are also widespread in natural environments, including soil, dust, air, and fresh water, wherever one-carbon compounds are abundant. Because of their ability to metabolize various plant decomposition compounds such as methylated compounds, methylotrophic bacteria play an important ecological role in the environmental carbon cycle. Although *Methylobacterium* strains have been frequently found on plant tissues (19), there is no previous evidence of symbiotic association of such microorganisms with plants. We demonstrated here that a group of *Methylobacterium* strains, identified as the new species "*M. nodulans*," are able to form nitrogen-fixing nodules on the roots of leguminous plants. However, this case may not be unique since we recently learned that CB376, a nodulating photosynthetic strain from *L. bainesii* (14), could be classified in the *Methylobacterium* genus on the basis of its 16S rRNA sequence (W. Heumann, personal communication).

A polyphyletic origin of rhizobia versus a monophyletic origin of common nodulation genes suggests that rhizobia have evolved through acquisition of nodulation functions in different bacterial branches susceptible to adaptation to different legume environments. "*M. nodulans*" is a facultative methylotroph, a unique property among rhizobia, raising the question of the role of methylotrophy in *Crotalaria*-"*M. nodulans*" symbiosis. We are currently starting a genetic analysis to understand why *Methylobacterium* strains are specifically associated with some particular legumes.

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ARTICLE 18

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Competing interests statement

The authors declare that they have no competing financial interests.

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Bacteriophytochrome controls photosystem synthesis in anoxygenic bacteria

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Plants use a set of light sensors to control their growth and development in response to changes in ambient light. In particular, phytochromes exert their regulatory activity by switching between a biologically inactive red-light-absorbing form (Pr) and an active far-red-light absorbing form (Pfr)[1,2]. Recently, biochemical and genetic studies have demonstrated the occurrence of phytochrome-like proteins in photosynthetic and non-photosynthetic bacteria[3–7]—but little is known about their functions. Here we report the discovery of a bacteriophytochrome located downstream from the photosynthesis gene cluster in a *Bradyrhizobium* strain symbiont of *Aeschynomene*. The synthesis of the complete photosynthetic apparatus is totally under the control of this bacteriophytochrome. A similar behaviour is observed for the closely related species *Rhodopseudomonas palustris*, but not for the more distant anoxygenic photosynthetic bacteria of the genus *Rhodobacter*, *Rubrivivax* or *Rhodospirillum*. Unlike other (bacterio)phytochromes, the carboxy-terminal domain of this bacteriophytochrome contains no histidine kinase features. This suggests a light signalling pathway involving direct protein–protein interaction with no phosphorelay cascade. This specific mechanism of regulation may represent an important ecological adaptation to optimize the plant–bacteria interaction.

We have previously identified an unexpected haem oxygenase gene close to the photosynthesis gene cluster in the symbiotic photosynthetic *Bradyrhizobium* ORS278 strain[8]. The haem oxygenase opens the haem ring to form the linear tetrapyrrole biliverdin, the first intermediate in the synthesis of phytochrome chromophore[9]. This prompted us to search for the possible involvement of a bacteriophytochrome in the regulation of the synthesis of the photosynthetic apparatus in this species and other anoxygenic photosynthetic bacteria.

Sequencing the downstream region from the haem oxygenase gene of *Bradyrhizobium* ORS278 reveals, in the opposite orientation, an open reading frame (ORF) that encodes a polypeptide containing 724 amino acids (Fig. 1a). Its amino-terminal region displays a strong similarity to the chromophore-binding domain of

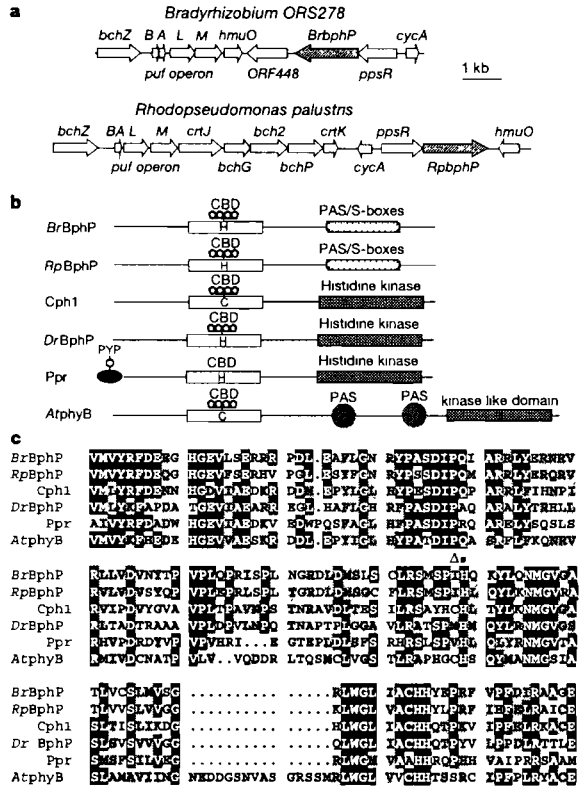

Figure 1 Molecular characterization of phytochromes. **a**, Arrangements of genes located downstream from the photosynthesis gene cluster in *Bradyrhizobium* ORS278 and *Rps palustris* *BrbphP*, *Bradyrhizobium* bacteriophytochrome photoreceptor, *RpbphP*, *Rps palustris* bacteriophytochrome photoreceptor, *cycA*, gene encoding cytochrome c_2; *hmuO*, gene encoding haem oxygenase, *ppsR*, gene encoding the transcriptional factor PpsR. **b**, Domain structure of various phytochrome sequences. *Br*BphP, from *Bradyrhizobium* ORS278, *Rp*BphP, from *Rps palustris*; Cph1, from *Synechocystis* PCC6803, *Dr*BphP, from *D. radiodurans*; Ppr, from *R. centenum*; and *At*phyB, phytochrome B from *Arabidopsis thaliana*. CBD, chromophore binding domain, PAS, Per/Arnt/Sim repeats, PYP, photoactive yellow protein domain. **c**, Amino-acid sequence alignments of chromophore binding domains of *Br*BphP, *Rp*BphP, Cph1, *Dr*BphP, Ppr and *At*phyB. The open and filled triangles indicate the conserved Cys and His residues involved in chromophore attachment in plant phytochromes and in *Dr*BphP, respectively. Black background, identical residues; Grey background, similar residues.

bacterial and plant phytochromes (Fig. 1b), but lacks the conserved Cys residue expected to be the bilin ligand in plant phytochrome[1] (Fig. 1c). However, the adjacent His residue reported to serve as the bilin attachment site in *Deinococcus radiodurans* phytochrome (*Dr*BphP) is conserved[6]. Unlike the case in other bacteriophytochromes, the C-terminal region of this protein, named *Br*BphP for *Bradyrhizobium* bacteriophytochrome photoreceptor, is not related to the histidine kinase domain, as none of the four conserved motifs that make up the catalytic centre is present[10]. However, an S-box domain that corresponds to a strongly conserved region in PAS (Per/Arnt/Sim) domains[11] is present from amino acid 518 to 642 (Fig. 1b). These domains, which may be involved in protein–protein interactions, are present in a large family of sensor proteins including the plant phytochromes[12]. Immediately upstream of *BrbphP*, we identified an ORF encoding a 456-amino-acid protein homologous to the transcriptional factor PpsR (Fig. 1a). This protein represses the expression of bacteriochlorophyll (*bch*) and carotenoid (*crt*) genes and of light-harvesting II complex (*puc*) structural genes at high oxygen tension or high light intensity in anaerobic purple bacteria[13,14].

Purified recombinant *Br*BphP apoprotein was tested for *in vitro* attachment with various purified bilins by zinc-induced autofluorescence. The apoprotein covalently binds phycocyanobilin (PCB), phycoerythrobilin (PEB) and also biliverdin (BV), a bilin that cannot assemble with plant phytochromes[15] but only with bacteriophytochromes[16]. The Pr form is produced in the dark immediately after addition of the chromophore, but is predominantly transformed into the Pfr form with a half-time of 30 min in a non-photochemical process (data not shown). Only the chromoproteins reconstituted with BV or PCB are photoactive and display reversible characteristic photoconversion between the Pr and Pfr forms (Fig. 2). We note that the Pfr form of the BV-bacteriophytochrome presents the highest wavelength of maximum absorption (λ_{max} = 750 nm) observed to date[16] (Fig. 2). In agreement with the absence of a histidine kinase domain, no autophosphorylation could be observed in kinase assays *in vitro* for the active *Br*BphP using either red or far-red illumination.

To prove the functional involvement of *Br*BphP in the synthesis of the photosynthetic apparatus, *Bradyrhizobium* ORS278 cells were grown under continuous illumination with low irradiance of different wavelengths preferentially absorbed by the Pfr and Pr forms, or by the bacteriochlorophyll molecules (870 nm). Bacteriochlorophyll fluorescence was used as a specific marker of the presence of the photosynthetic apparatus (Fig. 3a). The wavelength dependence of photosystem synthesis observed *in vivo* (Fig. 3b) is characteristic of the absorption of the Pfr form of the BV-reconstituted *Br*BphP with an optimum close to 750 nm. This suggests that BV may be the chromophore of the native form of *Br*BphP. This experiment also indicates that Pr is the active form of this bacteriophytochrome. The enhancement of photosystem synthesis by the Pr form can be reversed to the level observed in the dark by applying 660-nm illumination at an irradiance approximately six times that of the 740-nm illumination, demonstrating the photoreversibility of the phytochrome effect. A further proof of the requirement of far-red illumination for the synthesis of photosynthetic apparatus was obtained by the precise and direct determination of the amount of light-harvesting complexes and reaction centres, by measuring the absorption spectrum and the light-induced photooxidation of

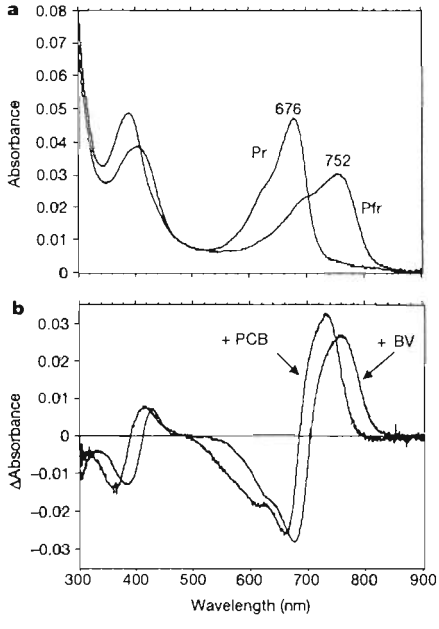

Figure 2 *In vitro* assembly of *Bradyrhizobium* bacteriophytochrome. **a**, Absorption spectra of Pr and Pfr forms of *Bradyrhizobium* bacteriophytochrome reconstituted with biliverdin (BV). The Pr spectrum was measured following far-red illumination (λ > 740 nm). The Pfr spectrum was derived from a spectrum recorded under 660-nm illumination assuming a Pfr/Pr ratio of 0.68. **b**, Difference spectra between the Pfr and Pr forms of *Bradyrhizobium* bacteriophytochrome reconstituted with BV or phycocyanobilin (PCB).

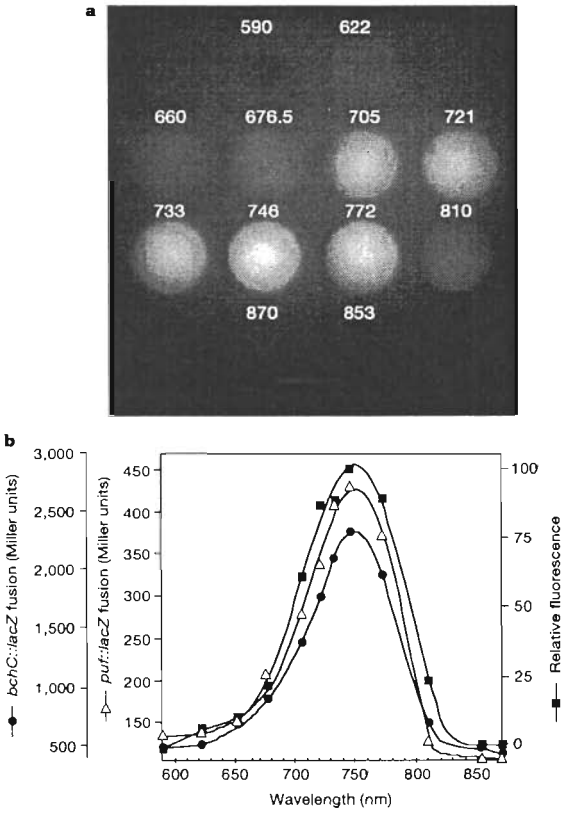

Figure 3 Action spectra for photosystem synthesis. **a**, Image of the long-wavelength fluorescence emission of bacteriochlorophyll of *Bradyrhizobium* cells grown on a Petri dish under light of different wavelengths (see Methods). **b**, Wavelength dependence of the synthesis of photosynthetic apparatus (filled squares) and expression of a *bchC*::*lacZ* (filled circles) and *puf*::*lacZ* (open triangles) fusions.

cytochrome, respectively, of intact cells (Fig. 4a). Furthermore, the expression of the *puf* operon and of the *bchC* gene—which encode the core proteins of the photosystem and an enzyme involved in bacteriochlorophyll synthesis, respectively—exhibited a wavelength dependence similar to the absorption spectrum of the Pfr form (Fig. 3b). This series of experiments shows that *Br*BphP controls the expression of various photosynthesis genes.

Proof of the involvement of *Br*BphP in the regulation of the photosystem synthesis by light was obtained with a null mutant of *Br*BphP generated by homologous-gene replacement. This mutant displays very low expression of the photosystem independent of the growth conditions (Fig. 4a). Altogether, these results give the molecular basis of the unexplained regulation of the photosynthetic apparatus synthesis by the light quality described for a symbiotic photosynthetic bacterium[17].

To determine whether this system of regulation is widespread among anoxygenic photosynthetic bacteria, we analysed the behaviour of various purple bacteria belonging to the α or β subclass *vis-à-vis* the light quality. No significant effect of the light quality is observed on the synthesis of the photosynthetic apparatus of cells of *Rhodobacter* (*Rba.*) *sphaeroides*, *Rba. capsulatus*, *Rhodospirillum centenum* and *Rubrivivax gelatinosus*. In contrast, *Rhodopseudomonas* (*Rps.*) *palustris* exhibits a behaviour identical to that observed in *Bradyrhizobium* ORS278 strain. As an example, Fig. 4b shows a typical result for *Rps. palustris* and *Rba. capsulatus*. It is clear that the accumulation of the photosynthetic apparatus, which confers the red colour to the bacteria, requires illumination by 740-nm light for cells of *Rps. palustris* but not for *Rba. capsulatus*. The wavelength dependence of the expression of the photosynthetic apparatus of *Rps. palustris* is similar to that reported in Fig. 3 for *Bradyrhizobium* ORS278 (data not shown). This implies that the photosynthesis genes are also controlled by a bacteriophytochrome in *Rps. palustris*. In agreement with this, scanning the genomic database of *Rps. palustris* revealed the presence of a bacteriophytochrome gene homologous to *BrbphP* (Fig. 1b). This gene is adjacent to a *ppsR* gene and close to the photosynthesis gene cluster in an arrangement very similar to *Bradyrhizobium* (Fig. 1a). The presence of the gene coding for the transcriptional factor PpsR adjacent to the gene of the bacteriophytochrome in both photosynthetic bacteria (Fig. 1a) suggests that this protein could act as a regulator of the light signalling pathway. To test this hypothesis, we disrupted the *ppsR* gene of *Bradyrhizobium* ORS278. In contrast to the wild-type strain, the synthesis of the photosynthetic apparatus remained at a high level in the dark or under 660-nm illumination in a null *ppsR* mutant (Fig. 4a), demonstrating that PpsR blocks the transcription of photosynthetic apparatus proteins in *Bradyrhizobium*. As the Pr form of *Br*BphP antagonizes the repressive activity of PpsR, we propose that these two proteins belong to the same light regulatory pathway, with *Br*BphP acting as the first and PpsR as the last element. Whether PpsR is the direct and only target of *Br*BphP has yet to be determined.

This series of experiments shows that *Rps. palustris* and photosynthetic *Bradyrhizobium* from *Aeschynomene* possess a sophisticated regulatory system where the synthesis of the entire photosynthetic apparatus is initiated by far-red light via the action of the Pr form of a bacteriophytochrome. This raises the question of why such a mechanism of regulation is only found in these two phylogenetically closely related species. A particular ecological niche and/or specific functional constraints may have favoured the establishment of this mechanism probably before their divergence. Such conditions are found in the case of photosynthetic *Bradyrhizobium* strains, which possess the exceptional capacity of nodulating both roots and stems of plants of the genus *Aeschynomene*[17]. In their stem symbiotic state, these bacteria grow below a layer of chlorophyll-containing cells, which absorbs preferentially blue and red light but transmits far-red light. Consequently, thanks to the control via a bacteriophytochrome, the light regulation of the photosystem synthesis allows the bacteria to switch rapidly from dark heterotrophy during root symbiosis to the more energetically favourable photoheterotrophy for stem symbiosis. As the bacterial photosynthetic activity is essential to the efficiency of the stem symbiosis[8], this mechanism of regulation may represent an important ecological adaptation to optimize the plant–bacteria interaction. In the case of *Rps. palustris*—which possesses some *nod* genes but has not yet been shown to interact with plants—it remains to be determined whether the light regulation of its photosystem synthesis is linked to specific growth conditions or to the conservation of an ancestral character. □

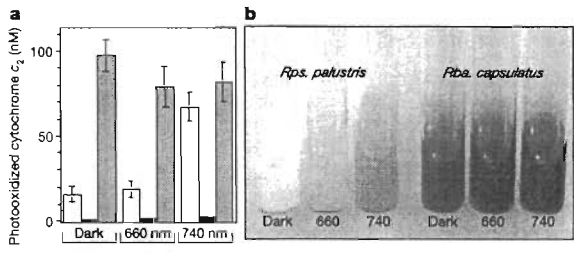

Figure 4 Effect of illumination on photosynthetic activity and pigmentation. **a**, Photosynthetic activity of *Bradyrhizobium* ORS278 wild-type strain (unshaded), 278Δ*BrbphP* (black) and 278Δ*ppsR* (grey) strains grown in the dark, under 660-nm and 740-nm continuous illumination provided by LEDs. The data represent the mean of four experiments (errors bars indicate ±s.d.). **b**, Pigmentation of *Rps. palustris* and *Rba. capsulatus* cells after growth under complete darkness or continuous red light (660 nm) or far-red light (740 nm).

Methods

Bacterial strains and growth conditions

Bradyrhizobium sp. strains were grown in a modified YM-agar medium[8]. *Rps. palustris*, *Rba. sphaeroides*, *Rubrivivax gelatinosus*, *Rba. capsulatus* and *Rhodospirillum centenum* were grown in Hutner medium[18]. All the strains were cultured in Petri dishes at 30 °C in either complete darkness or continuous red (660 nm) or far-red (740 nm) light provided by light-emitting diodes (LEDs).

Wavelength dependence determinations

Bradyrhizobium cells, homogeneously inoculated on solid modified YM medium in a Petri dish, were grown for 6 d under continuous illumination provided by a series of LEDs of different peak wavelengths between 590 and 870 nm. Each LED illuminated a 3.5-cm^2 area. Irradiance and spectra were assessed using a spectroradiometer. Half-peak bandwidth was below 25 nm for all wavelengths. Irradiance was adjusted at 6.6 μmol photons per m^2 per s to be below saturation for all tested wavelengths. The relative amounts of photosynthetic apparatus in the different illuminated areas were quantified by imaging bacteriochlorophyll fluorescence emission (above 800 nm) excited by blue-green illumination over the Petri dish using a cooled charge-coupled-device camera. The reporter gene *lacZ* was previously inserted into the *pufL* and *pufM* genes of ORS278[8]. For *lacZ*::*bchC* fusion, the *lacZ-kan*[r] cassette[19] was inserted into the unique *SalI* site of the *bchC* gene. Cells grown under the illuminated areas were resuspended in 3 ml of water, and β-galactosidase activity of *Bradyrhizobium* ORS278 strains harbouring the *bchC*::*lacZ* fusion or *puf*::*lacZ* fusion was measured as described[20].

Absorption and light-induced absorption change measurements

Light-induced absorption changes in intact cells or reconstituted *Br*BphPs were performed as previously described[21]. The amount of the photosynthetic apparatus in intact cells was determined by the measurement of the photooxidized cytochrome, the electron donor to the photosynthetic reaction centre, detected by absorbance change at 422 nm after a saturating flash. Absorption spectra of intact cells of the various species were recorded with a Cary 50 spectrophotometer.

Expression, purification and activation of *Br*BphP

BrbphP was amplified by polymerase chain reaction (PCR) from genomic DNA using the primers: 5′-AGGGGAGCCATATGCCCG TTCCGCTGACGACGCCA-3′ and 5′-GCGGGCGGCTCTTCC GCAACCCTCCTCGCTCTGCGAGCGATACCA-3′. PCR product was double digested with *NdeI* and *SapI* and ligated into the pTYBI vector (NEB) for expression in *Escherichia coli*. The *Br*BphP–intein fusion protein was purified using a chitin affinity column and self-cleaved by 30 mM dithiothreitol at 4 °C. Holo-*Br*BphP was

assembled with various bilins as described[15]. Spectra were obtained after saturating red (660 nm) and far-red (740 nm) irradiations. *In vitro* kinase assays for the active *Br*BphPs were performed as described[5].

Construction of *BrbphP* and *ppsR* mutant strains

To create *Brbph* and *ppsR* null mutants, the *lacZ-kan*[r] cassette[19] was inserted respectively in the *Xho*I site of *BrbphP* and the *Bgl*II site of *ppsR*. The constructions were introduced in the pJQ200 suicide vector[22] and delivered by conjugation into the ORS278 strain as previously described[8]. Double recombinants were selected on sucrose and confirmed by PCR.

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Competing interests statement

The authors declare that they have no competing financial interests.

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GenBank accession codes for the (bacterio)phytochrome sequences are AF182374 (*Bradyrhizobium* ORS278), AB00139 (*Synechocystis* PCC 6803), AAF12261 (*D. radiodurans*), AF06452[7] (*R. centenum*), X17342 (*Arabidopsis thaliana*). The genomic organization of *Rps. palustris* was deduced from the genome database at http://spider.jgi-psf.org/JGI_microbial/html/.

corrigendum

Nuclear translocation and transcription regulation by the membrane-associated guanylate kinase CASK/LIN-2

Yi-Ping Hsueh, Ting-Fang Wang, Fu-Chia Yang & Morgan Sheng

Nature **404**, 298–302 (2000).

In this Letter, we numbered some nucleotides for the upstream region of the *reelin* gene incorrectly. The Reelin-luc construct contains an upstream region of the *reelin* gene corresponding to nucleotides 157700–158620 of human BAC clone AC002067, instead of nucleotides 3700–4620. This does not affect any of the results or conclusions of the paper. We thank A. M. Goffinet, D. Grayson, K. Mendra and T. Curran for alerting us to this mistake. □

erratum

Origins and estimates of uncertainty in predictions of twenty-first century temperature rise

Peter A. Stott & J. A. Kettleborough

Nature **416**, 723–726 (2002).

On page 725 of this Letter, the words 'predicts[13] fThur lglk al seasitevier' were corrupted. They should read 'predicts[13]. This lack of sensitivity' □