Characterization of a New Xylanolytic Bacterium, 
Clostridium xylanovorans sp. nov.

T. MECHICHI, M. LABAT, J.-L. GARCIA, P. THOMAS, and B. K. C. PATEL

1Laboratoire ORSTOM de Microbiologie des Anaérobies, Université de Provence, Marseille, France
2Département de Biologie, Université de la Méditerranée, Marseille, France
3School of Biomolecular and Biomedical Sciences, Faculty of Science, Griffith University, Brisbane, Queensland, Australia

Received March 31, 1999

Summary

A new xylanolytic bacterium designated strain HESPIT (T = type strain) was isolated from a methanogenic digester. Strain HESPIT was a motile, rod shaped, spore-forming bacterium, which possessed a Gram-positive type cell wall. Glucose, fructose, lactose, trehalose, maltose, raffinose, sucrose, xylan, mannitol, cellobiose, galactose, mannose, melibiose, ribose were fermented to produce, acetate, butyrate, H2, CO2, formate, isobutyrate, and ethanol. Fumarate was fermented to acetate. Glycerol and methanol were also utilized. Sulfate, thiosulfate, nitrate, sulfur and fumarate were not used as electron acceptors. Strain HESPIT had a G+C content of 40 mol% and grew optimally at 37 °C and pH 7 on a fructose containing medium. Phylogenetically, strain HESPIT was most related to Clostridium aminoullericum (similarity of 94%) than to C. populeti, C. herbiuorans and Eubacterium xylanophilum (average similarity of 92%), all members of subcluster XIVa of the low G+C containing Gram-positive branch. However, strain HESPIT shared little phenotypic and genotypic traits with C. aminoullericum and on the basis of this and phylogenetic evidence, we propose to tentatively designate strain HESPIT as a new species of the genus Clostridium, Clostridium xylanovorans sp. nov. The type strain is HESPIT (= DSM 12503).

Key words: Clostridium xylanovorans – xylan – methanogenic digester – phylogeny – fermentation

Introduction

Xylan, one of the most important plant cell wall components, is a heteropolymer composed primarily of β-1-4-linked xylose with various amounts of arabinose, glucose, galactose, and other sugars as side group depending on the plant source. Of the numerous diverse microbial species reported, their representation is perhaps the most dominant in the phylum that contains the low G+C Gram-positive clostridial group. C. aldrichi (YANG et al., 1990), C. aerotolerans (VAN GYLSWY and VAN DER TOORN, 1987), C. cellulovorans (SLEAT et al., 1984), C. lentocellum (MURRAY et al., 1986), C. polysaccharolyticum (VAN GYLSWY et al., 1983), C. populeti (SLEAT and MAH, 1985), and C. xylanolyticum (ROGER and BAECKER, 1991) are such examples of the diversity found in this phylum. In this paper, we provide evidence that a new anaerobic xylanolytic bacterium isolated from an olive mill wastewater treatment digester, and designated HESPIT, has phenotypic, phylogenetic and genomic characteristics distinct from other xylanolytic bacteria described so far. Based on this evidence we propose that this strain be tentatively placed in the genus Clostridium as Clostridium xylanovorans sp. nov until the time of revision of the phylum.

Materials and Methods

Source of strains: Strain HESPIT was enriched from samples collected from an anaerobic olive mill wastewater-fed methanogenic digester (maintained at 37 °C) in Tunisia. Samples were collected and stored under anaerobic conditions in an O2-free N2 atmosphere in serum bottles and subsequently transported to the laboratory at ambient temperature.

Enrichment, isolation and cultivation: A basal medium used for enrichment, isolation, and cultivation was prepared by anaerobic techniques described by HUNGATE (1969) and modified for use with syringes (Macy et al., 1972; Miller and Wollin, 1974). The basal medium contained (per liter of deionized water): 1 g of NH4Cl, 0.3 g of K2HPO4, 0.3 g of KH2PO4, 0.723-2020/99/22/03-366 $ 12.00/0

Fonds Documentaire ORSTOM
Cote: B*19358 Ex: 1
DNA base composition: The G+C content of DNA was determined at DSM-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. The DNA was isolated and purified by chromatography on hydroxyapatite, and the G+C content was determined by using high-performance liquid chromatography (HPLC) as described by Misbah et al., (1989). Nonmethylated lambda DNA (Sigma) was used as the standard.

16S rDNA studies: DNA was extracted from the isolate as described previously (Redburn and Patel, 1993; Andrews and Patel, 1996). The universal primers R1 and R1d were used to obtain a PCR product of approximately 1.5 kb corresponding to base positions 14 to 1539 based on E. coli numbering of the 16S rDNA (Winiker and Woeser, 1991). A 50-pl reaction contained 1–20 ng of genomic DNA, 1 μM of each primer, 5 μl of 10 x buffer, 200 μl of dNTP, 3.5 mM of MgCl2, and 2.5 U of *Taq* polymerase (Promega). PCR was carried out by an initial denaturation at 94 °C for 7 min, then 29 cycles of annealing at 53 °C for 2 min, extension at 72 °C for 4 min, denaturation at 94 °C for 1 min, and finally an extension cycle of 53 °C for 2 min and 72 °C for 20 min.

PCR products were purified using a QIAquick Kit (Qiagen). DNA concentration of purified PCR product was estimated by comparison with the Low Mass Ladder (Gibco BRL) on an agarose gel containing ethidium bromide. QIAquick purified PCR products were sequenced using the ABI PRISM Dye Terminator Cycle Sequencing Kit containing AmpliTaq FS DNA polymerase and ABI 373A sequences. A 10-μl reaction contained 5 ng of PCR product, 4 μl of cycle sequencing reaction mix, 3.2 pmol of primer, and 2.5 μg of BSA. Thermal cycling was carried out using a RapidCycler (Idaho Technology) at a temperature transition slope of 2, an initial denaturation of 94 °C for 15 sec, then 25 cycles of denaturation at 94 °C for 0 sec, annealing at 50 °C for 10 sec, extension at 60 °C for 3 min.

The new sequence data that was generated was aligned, an almost full length consensus 16S rRNA gene sequence assembled and checked for accuracy manually using the alignment editor, a2e (Maidak et al., 1997). These were compared with other sequences in the GenBank database (Benson et al., 1993) using BLAST (Altschul et al., 1997), and in the Ribosomal Database Project, version 7.0 using SIMILARITY_RANK and SUGGEST_TREE (Maidak et al., 1997). If necessary, reference sequences most related to our newly generated sequence were extracted from these databases and aligned. Positions of sequence and alignment uncertainty were omitted from the analysis. Pairwise evolutionary distances based on 1280 unambiguous nucleotides were computed using dnadist (Jukes and Cantor) and neighbor-joining programs that form part of the PHYLIP suite of programs (Felsenstein, 1993). TREECON was used extensively for bootstrap analysis (Van de Peer and De Wachter, 1993).

Results

Morphology

Cells of strain HESPIT were rod-shaped (0.4–0.8 μm x 7–14 μm) and usually occurred singly. The strain stained Gram positive and thin section of cells examined under an electron microscope revealed a single thick tri-layered cell wall structure typical of Gram-positive type walls (Fig. 1). The presence of terminal spherical spores distended the cells. Cells were motile and negatively stained cell preparations showed the presence of subpolar to laterally inserted flagella.
FICHE DESCRIPTIVE

Auteur(s) : Mechichi T., Labat M., Garcia J.-L., Thomas P., Patel B.K.C.

Titre original : Characterization of a new xylanolytic bacterium, Clostridium xylanovorans sp. nov.


Titre en Français : Caractérisation d’une nouvelle bactérie xylanolytique, Clostridium xylanovorans sp. nov.

Mots-clés matières : Clostridium xylanovorans, phylogénie, taxonomie, anaérobiose, xylane, fermentation (10 au plus)

Résumé en Français :
(150 mots maximum)

Plan de classement : Monde végétal et Animal - Fermentations
Physiology and G+C content

Strain HESP1T was a strictly anaerobic chemoorganotrophic bacterium. The temperature range for growth was between 25 and 42 °C, and the optimum 37 °C. The pH range for growth was between 6-8 and the optimum pH 7. Strain HESP1T was inhibited in the presence of 1.5% NaCl but could grow in the presence of 0 to 1% NaCl. Strain HESP1T grew in the presence of cellobiose, fructose, galactose, glucose, lactose, maltose, mannitol, mannose, melibiose, raffinose, ribose, sucrose, trehalose, xylan, glycerol, methanol and fumarate but not on xy-

---

**Fig. 1.** Transmission electron micrograph of the cell wall ultrastructure of strain HESP1T showing the presence of cytoplasmic membrane (cm) and a single thick tri-layered electron dense cell wall composed of the inner layer (il) adjacent to the cytoplasmic membrane, the middle layer (ml) and the outer layer (ol). Part of the spore (sp) of the strain can also be seen. The cell wall of the spore has the same structure as the bacterial cell wall, and is typical for endospores. Bar = 0.2 µm.

---

**Fig. 2.** Unrooted dendrogram based on 16S rRNA sequence data indicating the phylogenetic position of Clostridium xylanovorans (strain HESP1T = DSM 12503T) within the radiation of representatives of subcluster XIVa of the low G+C containing gram positive bacteria. Sequences represented in subcluster XIVb (indicated as a triangle) were used as outgroup and include Clostridium lentocellum and Clostridium propionicum. The clusters are defined as suggested by Collins et al., 1994. All the sequences used in the analysis were obtained from the RDP (Ribosomal Database Project), version 7.0 (Maidak et al., 1997). Bootstrap values, expressed as a percentage of 100 replications are shown at the branching points. Only values above 90% were considered significant and therefore reported. Scale bar indicates 2 nucleotide substitutions per 100 nucleotides.
lose, sorbose, sorbitol, myo-inositol, arabinose, arabitol, cellulose, formate, acetate, propionate, butyrate, valerate, crotonate, malonate, malate, lactate, citrate, succinate, ethanol, propanol, isopropanol, butanol, isobutanol, biotypcase, yeast extract, casamino acids, gelatin and peptone.

The major fermentation end-products of glucose, fructose, lactose, trehalose, maltose, raffinose, sucrose, xylan, mannitol, cellobiose, galactose, mannos, melibiose and ribose were acetate and butyrate and minor products were H₂, CO₂, formate, isobutyrate, and ethanol.

Fumarate was fermented to acetate. None of the electron acceptors tested were utilized.

The DNA G+C base composition of strain HESPIT was 40 mol% as determined by HPLC method.

Phylogeny

Using 12 primers, we determined an almost complete 16S rRNA gene sequence of strain HESPIT consisting of 1533 bases corresponding to E. coli positions 14 to 1539 (nomenclature of WINKER and WOESE, 1991). Phylogenetic analysis revealed that strain HESPIT was a member of low G+C containing Gram-positive branch as defined by COLLINS et al. (1994) and grouped with members of the subcluster XIVa. The closest relative was Clostridium aminovalericum (similarity of 94%) with E. xylanophilum, C. populeti and C. polyzaccharolyticum slightly distantly related (average similarity of 92%).

Fig. 2 represents a dendrogram generated by the neighbor-joining method (FELSENSTEIN, 1993) from the JUKES and CANTOR evolutionary distance matrix (JUKES and CANTOR, 1969) depicting the relationship of strain HESPIT with representative members of the subcluster XIVa.

Detailed analysis of the 16S rRNA gene from position 450 to 483 (E. coli nomenclature of WINKER and WOESE, 1991) revealed a unique stretch of 29 nucleotides in strain HESPIT (Fig. 3a) but a reduced 9 nucleotide stretch in all other members of the subcluster XIVa (Fig. 3b) and 35 nucleotides in E. coli (Fig. 3c). In general, a reduced length is present in a vast majority of the members of the clusters of the low G+C containing Gram positive bacteria, with the exception of members of cluster IX.

Nucleotide sequence accession number

The 16S rRNA gene sequence of strain HESPIT has been deposited in the GenBank database under accession number AF116920.

Discussion

The ability to degrade xylan in mesophilic environments is widely distributed amongst anaerobic bacteria and includes some members of the genera Clostridium (VAREL et al., 1995), Eubacterium, Fibrobacter, Butyribrio, Selenomonas (COTTA et al. 1995) and Prevotella (AVGUSTIN et al., 1995). These bacteria have been isolated from a variety of different ecosystems such as animal intestinal tract, anaerobic digester, sewage sludge and soil. The isolation of strain HESPIT from a methanogenic digester fed with olive oil mill wastewater extends the known habitat of xylan degrading anaerobic bacteria. This type of environment is rich in plant xylans, cellulos and aromatic compounds. Whether it is a dominating member of the digester can be undertaken by developing probes targeted against the unique region of the 16S rRNA of strain HESPIT (Fig. 3b) identified during the course of this investigation.

Strain HESPIT is an anaerobic, mesophilic, spore-forming, motile, rod-shaped bacterium which stains Gram-positive and has a Gram-positive type cell wall ultra-structure. It does not reduce sulfate and is a carbohydrate fermenter. Phylogenetic analysis indicated that strain HESPIT was a member of the subcluster XIVa (COLLINS et al. 1994) in the low-G+C containing Gram-positive bacteria. On this basis, its taxonomic relationship to other xylanolytic Clostridium species distributed in cluster I (C. cellulovorans, cluster III (C. aldrichii) and subcluster XIVb (C. lentocellum)) can be ruled out. Sub-cluster XIVa currently comprises of several cellulolytic and/or xylanolytic clostridial species including C. aerotolerans, C. celerecrezens, C. sphenooides, C. xylanolyticum, C. populeti and C. polyascharolyticum. The closest relative of strain HESPIT in subcluster XIVa was C. aminovalericum (similarity of 94%). Phenotypically, both strain HESPIT and C. aminovalericum (HARDMAN and
Stadtman, 1960] are rod-shaped, motile, sporulates and grow at mesophilic temperatures. However, \textit{C. aminovalericum} ferments a limited number of carbohydrates, has a G+C content of 33 mol\% and utilizes aminovalerate and peptides but strain HESPIT\textsuperscript{T} has a wider carbohydrate utilization spectrum, has a G+C content of 40\% and is unable to utilize aminovalerate and peptides (Table 1).

Based on the evidence presented and based on the current taxonomic guidelines, we propose that strain HESPIT\textsuperscript{T} be designated a new member of the genus \textit{Clostridium} in the subcluster XIVa. However, a major taxonomic revision for the members of the low G+C containing Gram-positive bacteria has been proposed. Under this proposal, cluster I is to be reserved for members of the redefined genus \textit{Clostridium} (Collins et al., 1994). Strain HESPIT\textsuperscript{T} is not a member of cluster I and hence this will mean a change in the genus name for strain HESPIT\textsuperscript{T} at a later date. An alternative is to propose that a new strain HESPIT is not a member of cluster \textit{HESPIT} and \textit{C. aminovalericum}. However, we believe that it would be premature to attempt this in this communication. Any revision should wait until formal discussions on taxonomic changes are completed. Accordingly, we propose that strain \textit{HESPIT} be considered a temporary member of the genus \textit{Clostridium}, \textit{Clostridium xylanovorans} sp. nov., in the subcluster XIVa until such time when the revision of cluster I has been completed.

Description of strain \textit{Clostridium xylanovorans} sp. nov.

\textit{Clostridium xylanovorans} (xy.la.no.vo'rans. Gr. n. xylanosum, xylan; L. v. vorare devour to eat, xylan-eating bacterium). Cells stain Gram positive, are rod-shaped and form terminal spherical spore which swell the cell. The cells are motile by means of subpolar to laterally inserted flagella.

Growth is strictly anaerobic and chemoorganotrophic. Cells grow in the temperature range between 25 and 42 °C, with an optimum at 37 °C. The pH range for growth is between 6-8 with an optimum of pH 7. NaCl concentrations in the range of 0 to 1% do not inhibit growth.

The isolate grows on cellobiose, fructose, galactose, glucose, lactose, maltose, mannitol, mannose, melibiose, raffinose, ribose, sucrose, trehalose, xylose, glycerol, methanol and fumarate but not on xylose, sorbose, sorbitol, myo-inositol, arabinose, arabinol, cellulose, formate, acetate, propionate, butyrate, valerate, crotonate, malonate, malate, lactate, citrate, succinate, ethanol, propanol, isopropanol, butanol, isobutanol, biotyrcase, yeast extract, casamino acids, gelatin and peptone.

The major end products from fructose, glucose, lactose, trehalose, maltose, raffinose, sucrose, xylan, mannol. cellobiose, galactose, mannose, melibiose and ribose fermentation are acetate and butyrate with H\textsubscript{2}, CO\textsubscript{2}, formate, and ethanol also produced. Fumarate is reduced to acetate.

The DNA base composition of strain HESPIT\textsuperscript{T} is 40 mol\% as determined by HPLC. The closest phylogenetic relative is \textit{C. aminovalericum}. The type strain is HESPIT\textsuperscript{T} (= DSM 12503).

Acknowledgements:
The financial assistance in part to BKCP from the Australian Research Council is gratefully acknowledged. Frederic Verhe is thanked for technical assistance.

References


Table 1. Characteristics of strain HESPIT\textsuperscript{T} and \textit{Clostridium aminovalericum}.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Strain HESPIT\textsuperscript{T}</th>
<th>\textit{C. aminovalericum}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell size</td>
<td>0.8-1.0x4-10 (\mu\m)</td>
<td>0.3-0.5x1.5-5.2 (\mu\m)</td>
</tr>
<tr>
<td>pH optima</td>
<td>7</td>
<td>nr</td>
</tr>
<tr>
<td>Temperature range</td>
<td>20-45 °C</td>
<td>25-45 °C</td>
</tr>
<tr>
<td>G+C content</td>
<td>40%</td>
<td>33%</td>
</tr>
<tr>
<td>Utilization of:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>glucose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>fructose</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>cellobiose</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>maltose</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>sucrose</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>trehalose</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>xylose</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Proteins\textsuperscript{1}</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Aminomaleterate</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Yeast extract (0.2%)</td>
<td>-</td>
<td>nr</td>
</tr>
</tbody>
</table>

Both, strain HESPIT\textsuperscript{T} and \textit{C. aminovalericum}, are motile, sporulating rods and have a temperature optimum of 37 °C.

\textsuperscript{1} This study. Culturing was performed in basal medium (pH 7) supplemented with the indicated substrates at 20 mM, and the results recorded after two weeks of incubation at 37 °C

\textsuperscript{2} According to the data of Hardman and Stadtman (1960).

\textsuperscript{3} Proteins tested were peptone, meat extract.

nr = not reported; + positive growth; - negative growth.


Corresponding author:
B. K. C. Patel, School of Biomolecular Sciences, Faculty of Science, Griffith University, Brisbane, Queensland 4111, Australia
Tel: +61-417-726 671; Fax: +61-7-5875 7656;
E-mail: bharat@genomes.sci.gu.edu.au