Plant Science, 39 (1985) 125—131
Elsevier Scientific Publishers Ireland Ltd.

THE STRUCTURAL *NIF* GENES OF FOUR SYMBIOTIC *ANABAENA AZOLLAE* SHOW A HIGHLY CONSERVED PHYSICAL ARRANGEMENT

C. FRANCHE[a] and COHEN-BAZIRE[b]

[a]*Laboratoire de Microbiologie, O.R.S.T.O.M., B.P. 1386, Dakar Sénégal (West Africa) and* [b]*Unité de Physiologie Microbienne, Département de Biochimie et Génétique Moléculaire, Institut Pasteur, 75724 Paris Cedex 15 (France)*

(Received August 24th, 1984)
(Revision received January 30th, 1985)
(Accepted February 18th, 1985)

Cloned DNA from *Anabaena* sp. PCC 7120 was used to determine the distribution of restriction sites around the *NifHDK* genes of endosymbionts extracted from four *Azolla* species. Although many of the restriction sites of the symbiotic *Anabaena nifHDK* genes differed from those of the free-living *Anabaena* sp. PCC 7120, three apparently identical restriction sites were found in and around the *nifD* region. The arrangement of *A. azollae* and *Anabaena* sp. PCC 7120 *nif H,D,K* genes appears similar, with *nifH* and *nifD* linked and *nifK* some distance away from *nifD*. The *A. azollae nifHDK* genes appear strongly conserved among the *Azolla* species examined, regardless of the geographical origin of the ferns.

Key words: Azolla; endosymbiotic *Anabaena azollae;* nitrogen-fixation genes

Introduction

The water fern of the genus *Azolla* contains a symbiotic heterocystous cyanobacterium, *A. azollae*, within specialized leaf cavities [1]. The endophyte can supply the association with its total nitrogen requirement by nitrogen fixation [2]. Thus, *Azolla* constitutes a potential nitrogen source in agriculture and its use as green fertilizer in rice culture is well documented [3]. Physiological and morphological aspects of the fern-cyanobacterial relationship have been described [2,4,5]. Nevertheless the difficulty of culturing *A. azollae* in vitro is a major obstacle to the study of this important symbiosis. Putative cultures of *A. azollae* isolates have been obtained [4—7], but no one has successfully re-infected sterile plants with cultured *Anabaena*.

Abbreviation: LB, Luria Broth.

Nitrogen fixation (*nif*) gene organization has been analysed in the free-living *Anabaena* sp. PCC 7120, using cloned *Klebsiella pneumoniae nif* gene probes; sequences homologous to the structural genes for nitrogenase (*nifK* and *nifD*) and nitrogenase reductase (*nifH*) were identified and cloned [8]. The physical map of these genes differs from that of *K. pneumoniae*: only *nifH* and *nifD* are contiguous; *nifK* is separated from *nifH-D* by 11 kilobase (kb) [9]. Recently, the arrangement of the structural *nif* genes was also examined in the unicellular cyanobacterium *Gloeothece* PCC 6909 and in the filamentous cyanobacterium *Calothrix* sp. PCC 7601 [10]. In *Gloeothece*, the *nif* structural genes are grouped whereas in *Calothrix nifK* is separated from *nifH-D* as it is in *Anabaena* sp. PCC 7120. To our knowledge, no information concerning the organization of the *nif* genes is available for a symbiotic cyanobacterium.

Using probes from the free-living *Anabaena* sp. PCC 7120, we compare in this work

0168-9452/85/$03.30
Printed and Published in Ireland

restriction sites around the structural *nif* genes coding for nitrogenase and nitrogenase reductase in four *A. azollae* symbionts extracted from different species of *Azolla* and in a cultured isolate of *A. azollae* obtained by Tel-Or et al. [7]. Preliminary results concerning the arrangement of these *nif* genes will be discussed.

Materials and methods

Cyanobacterial strains, plasmids and Azolla species

Cyanobacterial strains, plasmids and *Azolla* species are listed in Table I.

Media and growth conditions

Anabaena sp. PCC 7120 and the cultured isolate of *A. azollae* were grown in BG-11 medium [10]. Luria Broth (LB) was the complete medium used for *Escherichia coli* strains containing the plasmids [12]. Fronds of *Azolla* were grown in 2 l of the medium described by Roger and Reynaud [13]. Symbiotic *A. azollae* were extracted from sterilized *Azolla* fronds according to the procedure of Peters and Mayne [14].

DNA isolation procedures

Bacterial plasmid DNA was purified according to Humphreys et al. [15]. Total DNA was extracted from vegetative cells of free-living *Anabaena* or freshly isolated symbionts by the method of Quiviger et al. [16] and further purified by cesium chloride-ethidium bromide density gradient centrifugation [15].

Restriction endonuclease digestions and DNA electrophoresis

*Eco*RI, *Hind*III, *Hae*III, *Pvu*II, *Pst*I, *Sal*I and *Xba*I from Biolabs, *Bgl*II, *Xho*I and *Xba*I from BRL, *Bam*HI, *Ava*I and *Pvu*I from Boehringer Mannheim were used as recommended by the manufacturers. Restriction fragments were separated in 0.7% (w/v) agarose gels according to Quiviger et al. [16]. Lambda phage DNA fragments were used as molecular weight standards.

Preparation of hybridization probes

Purified plasmids were labelled with [α-^{32}P]dCTP (400 Ci/mmol, Amersham International) by nick translation [17]. The specific activity of the labeled DNA was approx. 10^8 cpm/μg of DNA.

Table I. Cyanobacterial strains, plasmids and *Azolla* species.

	Characteristics	Source/reference
Cyanobacterial strains		
PCC 7120	Free-living *Anabaena* sp.	[11]
A. azollae var. filiculoides	Cultured isolate of *A. azollae* extracted from *A. filiculoïdes*	[7]
Plasmids		
pAn207.8	pBR322 containing a 0.7-kb *Hind*III fragment within *nifK* of *Anabaena* sp. PCC 7120	[9]
pAn256.Δ1	pBR322 containing a 1.1-kb *Hind*III-*Eco*RI fragment within *nif*D of *Anabaena* sp. PCC 7210	[9]
pAn154.3	pBR322 containing a 1.8-kb *Hind*III fragment of which 100 b is in *nifD*, 900 b is all of *nifH* and 800 b is non-*nif* DNA	[9]
Azolla species		
A. caroliniana	Collected in United-States	Diara, H.F. (U.C.L.[a])
A. filiculoïdes	Collected in South Africa	Diara, H.F. (U.C.L.[a])
A. microphylla	Collected in Galapagos	Lumpkin, T.A.
A. mexicana	Collected in United States	Caudales, R.

[a] U.C.L., Université Catholique de Louvain (Belgium).

Southern hybrodization

DNA fragments were transferred from agarose gels onto nitrocellulose filters (Millipore HAWP, 0.45-μm pore size) and hybridized with heat-denatured probes (2×10^6 cpm/slot) following the technique of Southern [18]. Autoradiographs were obtained by exposing the filters for one to several days at −80°C to X-ray films.

Results

Sensitivity of A. azollae DNA to restriction endonucleases

The electrophoretic patterns of DNA fragments from *A. azollae* digested with eleven restriction endonucleases showed numerous cleavage sites for *Hind*III and *Eco*RI, fewer cleavage sites for *Pvu*II and *Xho*I and practically none for *Ava*I, *Bam*HI, *Hae*III, *Kpn*I, *Pvu*I and *Sal*I. The four freshly isolated symbionts gave closely similar patterns (data not shown). Based on these results, hybridization of the *Anabaena* sp. PCC 7120 *nifK*, *nifD* and *nifH* probes to total DNA from *A. azollae* was studied with *Hind*III, *Eco*RI, *Hind*III-*Eco*RI, *Pvu*II and *Xho*I DNA digests.

Homology between total DNA of A. azollae and the nitrogenase structural genes from Anabaena sp. PCC 7120

Hybridization with the nifK probe. Within each restriction endonuclease digest, the *nifK* probe hybridized to DNA fragments of identical size for the fresh isolates from *A. caroliniana*, *A. filiculoides*, *A. microphylla* and *A. mexicana* (Table II). Two small fragments of 2.7 and 0.9 kb were observed in the *Eco*RI digest; they are contained within a large *Pvu*II or *Xho*I fragment: 26 and 35 Kb, respectively (Fig. 1a). No hybridization band was observed in the *Hind*III digest (Fig. 1a, lane 4) suggesting that these fragments were too small to

Table II. Size of restriction endonuclease fragments of *A. azollae* DNA hybridizing with *Anabaena* PCC 7120 *nifK*, *nifD* and *nifH* probes. *A. azollae* strains were, respectively, the fresh isolates from *A. caroliniana* (a), *A. filiculoides* (b), *A. microphylla* (c), *A. mexicana* (d) and the cultured isolate from *A. filiculoides* (e). Total DNA from strain PCC 7120 (f) was used as positive hybridization control.

Restriction endonuclease	*Anabaena* strains	Size (kb)		
		nifK	*nifD*	*nifH*
*Eco*RI	a-b	2.7-0.9	1.55	18-16-1.9
	c	2.7-0.9	1.55	12.5-6-1.9
	d	2.7-0.9	1.55	12.5-6.5-1.9
	e	7.4	18-9	18-(8.1)-(4.2)[a]
	f	17	10	10-19
*Hind*III	a-b-c-d	*	2.6	(3)-2.6-2.1
	e	*	2.3	(4.7)-3.9-(2.6)-(1.75)
	f	0.7	2.6	1.9
*Hind*III-*Eco*RI	a-b-c-d	*	1.1	2.1-1.6-1.4
	e	*	2.3	3.9-(2.8)-(2.6)-(1.7)
	f	0.7	1.1	1.9
*Pvu*II	a-b-c-d	26	26	26-24-11.5
	e-f	ND[b]	ND	ND
*Xho*I	a-b-c-d	35	35	35
	e-f	ND	ND	ND

*No hybridization band observed.
[a] Number in parentheses: faintly hybridizing band.
[b] ND, not determined.

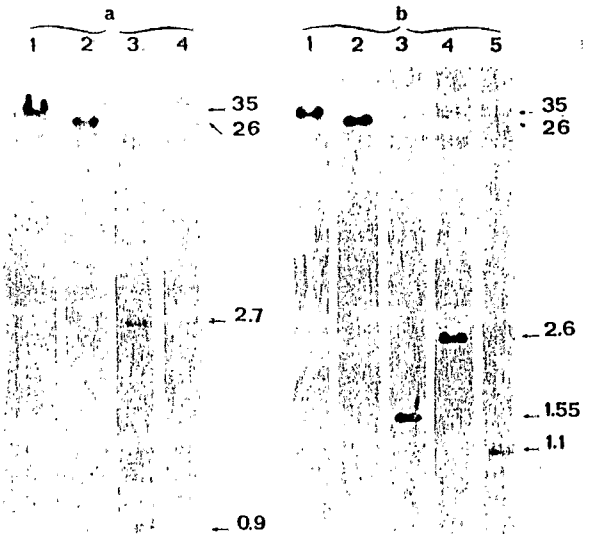

Fig. 1. Autoradiogram of ^{32}P-labeled *nifK* (a) and *nifD* (b) probes hybridized to *A. azollae* DNA extracted from *A. caroliniana*. Total DNA was digested respectively with *Xho*I (lane 1), *Pvu*II (lane 2), *Eco*RI (lane 3), *Hind*III (lane 4) and *Hind*III-*Eco*RI (lane 5). Sizes of DNA fragments are indicated in kb.

transfer well onto nitrocellulose. The same probe hybridized with one fragment of 7.4 kb in the *Eco*RI digest of the *A. azollae* var. filiculoides DNA and with fragments of, respectively, 17 kb and 0.7 kb in the *Eco*RI and *Hind*III DNA digests of the free-living *Anabaena* sp. PCC 7120, as expected based on the published map of the *nif* genes of this species [9].

Hybridization with the nifD probe. The homology detected between the *Anabaena* sp. PCC 7120 *nifD* probe and the DNA digests from the four symbiotic *A. azollae* was limited to a 1.1 kb *Eco*RI-*Hind*III fragment contained in a 1.55 kb *Eco*RI and a 2.6 kb *Hind*III fragment (Table II, Figure 1b). The same *Hind*III and *Hind*III-*Eco*RI hybridization bands were observed with the *Anabaena* strain PCC 7120. The hybridization of *nifD* to *A. azollae* var. filiculoîdes DNA produced a pattern different from any other strain (Table II). In the case of the fresh *A. azollae* isolates, the *nifD* probe hybridized to the same large *Pvu*II and *Xho*I fragments which hybridized with the *nifK* probe.

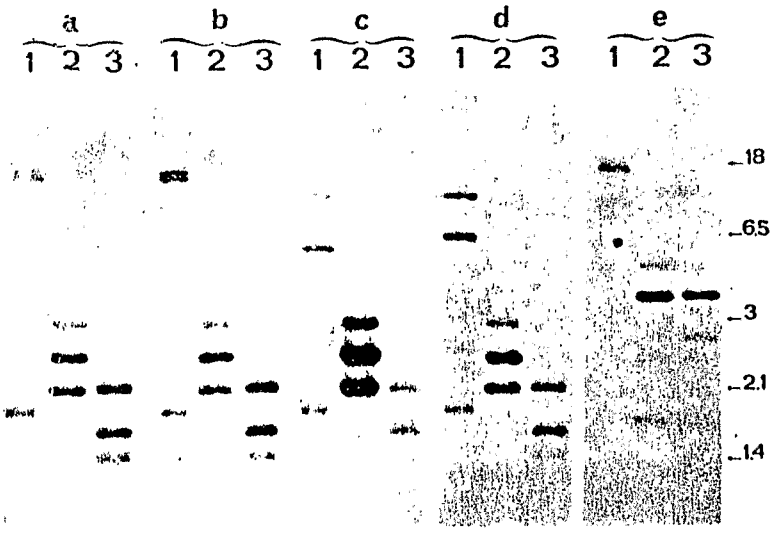

Fig. 2. Autoradiogram of ^{32}P-labeled *nifH* probe hybridized to *A. azollae* DNA extcted from *A. caroliniana* (a), *A. filiculoides* (b), *A. microphylla* (c), *A. mexicana* (d) and to free-living *A. azollae* var. filiculoides DNA (e). Total DNA was digested respectively with *Eco*RI (lane 1), *Hind*III (lane 2) and *Hind*II-EcoRI (lane 3). Sizes of DNA fragments are indicated in kb.

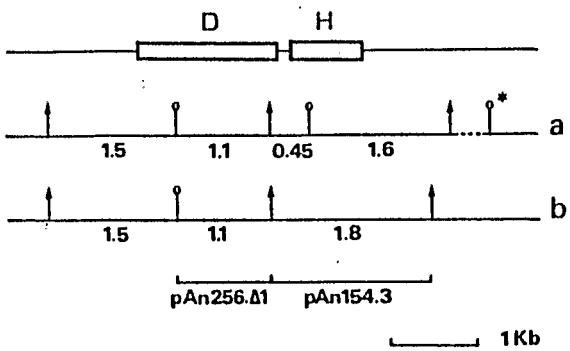

Fig. 3. Physical map of the *nifHD* genes of symbiotic *A. azollae* (a) and of *Anabaena* sp. PCC 7120 (b). Symbols for restriction endonuclease sites are: ↑*Hind*III and ρ*Eco*RI. *: This site could be 18, 16 or 1.9 kb away in *A. azollae* from *A. caroliniana* and from *A. filiculoïdes*, 12.5, 6 of 1.9 kb away in *A. azollae* from *A. microphilla* and 12.5, 6.5 or 1.9 kb away in *A. azollae* from *A. mexicana*.

Hybridization with the nifH probe. As shown in Table II, the *nifH* probe produced three hybridization bands with *Eco*RI (Fig. 2, lanes 1a, 1b, 1c and 1d), *Hind*III (Fig. 2, lanes 2a, 2b, 2c and 2d), *Hind*III-*Eco*RI (Fig. 2, lanes 3a, 3b, 3c and 3d) and *Pvu*II DNA digests of the four fresh *A. azollae* isolates. One strong hybridization fragment was obtained with the *Xho*I digest. Except in the case of *Eco*RI digests, the size of the homologous fragments within each restriction endonuclease digest was identical for the endophytes extracted from the four *Azolla* species (Table II). As already observed with the *nifK* probe, the *nifH* probe hybridized with *Eco*RI and *Hind*III DNA fragments of *A. azollae* var. filiculoides (Fig. 2, lanes 1e, 2e and 3e) and of the free living *Anabaena* PCC 7120 which differed in size from those of all the symbionts. The three probes (*nifK*, *nifD*, *nifH*) hybridized with the same *Pvu*II (26 kb) and *Xho*I (35 kb) fragments in all the symbiotic strains.

Discussion

A strong homology was observed between the cloned *nif* structural genes of *Anabaena* PCC 7120 and restriction digests of DNA of the *Anabaena* symbionts isolated from four different *Azolla* species. The data presented in Table II allow a comparison of size of the *Eco*RI, *Hind*III and *Eco*RI-*Hind*III restriction fragments that hybridized to the *nif* probes employed. In all four symbiotic *Anabaena* strains two small *Eco*RI fragments of 2.9 and 0.9 kb hybridized to the *nifK* probe, whereas only one large *Eco*RI fragment of 17 kb did so with an *Eco*RI digest of chromosomal DNA isolated from *Anabaena* 7120 (after growth with nitrate as the nitrogen source) as previously reported [9]. Therefore the DNA of each symbiotic *Anabaena* strain contains three *Eco*RI sites in and around the *nifK* gene, and these are not present in PCC 7120. One of them is directly within *nifK*; the other two are on either side of *nifK*, but much closer to this gene than the flanking *Eco*RI sites in PCC 7120.

The close proximity of *Hind*III and *Eco*RI restriction sites, together with the hybridization pattern of the corresponding restriction fragments to pAN256Δ1, allowed the construction of the physical map of the *nifD* region of all four symbiotic *A. azollae* strains (Fig. 3a) and this can be compared with that of *Anabaena* PCC 7120 (Fig. 3b). The *Hind*III sites within *nifD*, and those around this gene, are conserved in the two types of organisms, which also share an identical *Eco*RI site within *nifD*. However, there is an additional *Eco*RI site, not found in PCC 7120, just to the right of *nifD* in the DNA of the symbiotic strains.

Based on the *Hind*III and *Eco*RI sites in and around the *nifD* region, and the hybridization results obtained with the probe pAN-154.3, the location of *nifH* could be identified together with that of one *Hind*III restriction site to the right of *nifH* (Fig. 3a). However, this *Hind*III site is not identical in PCC 7210 and the symbiotic *Anabaena* strains; it is located 1.8 kb to the right of the conserved *Hind*III site of *nifD* in PCC 7120 and 2.1 kb to the right of the same *Hind*III site in the symbionts. Due to the *Eco*RI site within *nifH* in the symbiotic strains, additional

*Eco*RI sites to the right of this gene could be demonstrated. However, these sites are not identical in the four symbionts, except for that of 1.9 kb (Table II), since the resulting *Eco*RI fragments that hybridized to the *nifH* probe differed in size (Table II and legend to Fig. 3). Additional cloning experiments will be necessary to determine which of the *Eco*RI fragments are contiguous to the *Hind*III site on the right hand side of *nifH*.

On the basis of the restriction map shown in Fig. 3 and the complex hybridization patterns of pAN154.3 to *Eco*RI and *Hind*III digests and to *Eco*RI/*Hind*III double digests (Table II), we can conclude that there are two or more copies of *nifH* in the *A. azollae* genomes. Such *nifH* duplication also occurs in PCC 7120 and is located on a 19-kb *Eco*RI fragment [9]. Due to identical *Hind*III sites in *nifH* and the duplicated gene in PCC 7120 [9], this duplication is not evident in our *Eco*RI/*Hind*III digests. The function of the second *nifH* gene is unknown, but such duplication seems to be relatively common, since two or more *nifH* genes have been found in *Calothrix* PCC 7601 [10] and in several other nitrogen-fixing cyanobacteria (T. Kallas et al. unpublished results).

Hybridization of all three probes (*nif K*, *nif D*, *nifH*) to the large fragments obtained by *Pvu*II or *Xho*1 digestion of *A. azollae* DNA demonstrates that *nifK* and *nifDH* must be located within these fragments. However, cloning of the fragments will be necessary to establish with precision the physical map of the region between *nifK* and *nifDH*. Furthermore, we will examine whether this region is excised during heterocyst differentiation as has been observed for *Anabaena* PCC 7120 (R. Haselkorn, pers. commun.). In the latter strain, the excised fragment spans almost entirely the region between *nifK* and *nifDH*, i.e. a segment of about 11 kb.

Concerning the relatedness of the different *Anabaena* strains examined, we can draw the following conclusions: regardless of the geographical origin of the ferns assignable to the same *Azolla* subgenus (*Euazolla*), the restriction sites in the DNA of their symbionts in and around the *nifK* and *nifD* regions are identical, and only minor differences were observed in the *nifH* genes. These results suggest that the four symbionts from *A. caroliniana*, *A. filiculoides*, *A. mexicana* and *A. microphylla* are closely related, but that some evolutionary divergence has occurred. It would be of interest to perform the same type of hybridization experiments with *A. azollae* extracted from *A. nilotica* and *A. pinnata*. These species, morphologically very different from the *Euazolla* species, constitute the second *Azolla* subgenus named *Rhizosperma* [4].

Based on the completely different hybridization patterns observed with restriction digests of the cultured isolate of *A. azollae* (Tel-Or) from *Az. filiculoides* (Table II), we conclude that this organism is neither closely related to *Anabaena* PCC 7120 nor to any of the *Euazolla* symbionts. However, our conclusions concerning the relatedness of the various *Anabaena* strains examined, are only valid if we can assume that a divergence in a given chromosomal region, (here *nif*) is characteristic of that in total DNA. This assumption seems to be supported by the difference in sensitivity of total DNA of the Tel-Or isolate to eleven restriction endonucleases as compared to that of total DNA extracted from the four *Azolla* symbionts (unpublished results). Therefore, several possibilities concerning the association between *Az. filiculoides* and its cyanobacterial symbiont arise: (1) *Az. filiculoides* can form a symbiotic relationship with more than one species of *Anabaena*. (2) The organism isolated by Tel Or is only a minor constituent in the symbiosis of *Az. filiculoides* and its DNA was masked by that of the predominant symbiont extracted by us from this fern. (3) The cultured strain was a free-living *Anabaena* associated with the *Azolla* sample from which it was isolated. The analysis of DNA from additional *Az. filiculoides* samples (from different habitats) is required in order to establish which of these possibilities is correct.

Acknowledgements

We are very grateful to Dr. R. Haselkorn for valuable discussions and critical reading of the manuscript. We also thank Dr. P. Reynaud for advice, Dr. T. Kallas for the gift of plasmid pAn256.Δ1 and A.M. Castets for skillful technical assistance. This work was partly supported by Economic Eurpoean Community and ORSTOM under contract No. TSD-081-F(MR).

References

1 A.W. Moore, Bot. Rev., 35 (1979) 17.
2 G.A. Peters and B.C. Mayne, Plant Physiol., 53 (1974) 820.
3 T.A. Lumpkin and D.L. Plucknett, *Azolla* as a green manure, West View Press, 1982.
4 T.A. Lumpkin and D.L. Plucknett, Econ. Bot., 34 (1980) 111.
5 G.A. Peters, H.E. Calvert, D. Kaplan, O. Ito and R. Toia, Isr. J. Bot., 31 (1982) 305.
6 J.W. Newton and A.I. Herman, Arch. Microbiol., 120 (1979) 161.
7 E. Tel-Or, T. Sandovsky, D. Kobiler, H. Arad and R. Weinberg, G.C. Papageorgiou and L. Packer (Eds.), Photosynthetic Prokaryotes, in: Elsevier Science Publishing Co., New York, 1983, 303.
8 B.J. Mazur, D. Rice and R. Haselkorn, Proc. Natl. Acad. Sci. U.S.A., 77 (1980) 186.
9 D. Rice, B.J. Mazur and R. Haselkorn, J. Biol. Chem., 257 (1982) 13157.
10 T. Kallas, M.C. Rebière, R. Rippka and N. Tandeau de Marsac, J. Bacteriol., 155 (1983) 427.
11 R. Rippka, J. Deruelles, J. Waterbury, M. Herdman and R.Y. Stainer, J. Gen. Microbiol., 111 (1979) 1.
12 J.H. Miller, Experiments in Molecular Genetics, Cold Spring Harbor Laboratory (Ed.), 1972.
13 P.A. Roger and P.A. Reynaud, Oecol. Plant., 14 (1979) 75.
14 G.A. Peters and B.C. Mayne, Plant Physiol., 53 (1974) 813.
15 G.O. Humphreys, G.A. Willshaw and E.S. Anderson, Biochim. Biophys. Acta, 383 (1975) 457.
16 B. Quiviger, C. Franche, G. Lutfalla, D. Rice, R. Haselkorn and C. Elmerich, Biochim., 64 (1982) 495.
17 P.W.J. Rigby, M. Dieckmann, C. Rhodes and P. Berg, J. Mol. Biol., 113 (1977) 237.
18 E.M. Southern, J. Mol. Biol., 98 (9175) 503.
19 J.E. Gates, R.W. Fisher, T.W. Goggin and N.I. Azrolan, Arch. Microbiol., 128 (1980) 126.