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Partial Purification of a Cyanobacterial Membrane Protein with Amino Terminal Sequence Similarity to the *N*-Methylphenylalanine Pilins

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Cyanobacterial pilin was extracted from *Synechococcus* 6301 membranes using a detergent mixture comprising 1% Triton X-100, 1% Thesit® and 0.5% dodecyl β-D-maltoside. Partial purification of pilin from the crude extract was achieved by a single-step purification applying the Rotofor isoelectric focusing system. Up to 100-fold purification of pilin from the crude extract was achieved in a single run. SDS-PAGE analysis showed *Synechococcus* 6301 pilin migration with an apparent molecular weight of 11 kDa. The amino terminal sequence of the first 28 amino acid residues was identified. Alignment of the predicted sequence showed a 60–80% identity with amino terminal sequences of pilins from pathogenic gram-negative bacteria (enterobacteria). The apparent mass of *Synechococcus* 6301 pilin was, however, lower. The amino terminus of *Synechococcus* 6301 pilin, as with other pilins, has a high content of hydrophobic amino acids.

Fimbriae (pili) are protein filaments that extend from the cell surface of many bacteria and contain hundreds of identical protein subunits, pilin. These repeating pilins are helically arranged along the filament. Pilins are type specific and the apparent masses are in the range 8–20 kDa.[1]

The most well known pilin belongs to different species of pathogenic gram-negative bacteria (enterobacteria). Enterobacterial pili play a major role in mediating the adhesion of bacterial cells to tissue in the initiation and maintenance of infection.[2] The pili of *Pseudomonas aeruginosa*[3,4] belong to the biochemically and genetically best characterised class of enterobacterial pili which includes *Neisseria gonorrhoeae*,[5] *Moraxella bovis*,[6] *Moraxella nonliquifaciens*[7] and *Bacteroides nodusus*.[8]

The pilins of this group of bacteria show several biochemical similarities: (i) they are particularly rich in hydrophobic amino acids at the amino terminus, (ii) a 6–7 amino acid peptide is lost from the amino terminus of the pilin precursor in its conversion into mature protein, (iii) the amino terminus of the mature pilin is always the unusual amino acid *N*-methylphenylalanine. This class of pilus does, however, differ significantly from those found on various strains of *Escherichia coli*.[9]

Fimbriation in cyanobacteria was first reported by MacRae and co-workers in 1977.[10] Further studies have revealed the occurrence of pili or fimbriae in more than 20 cyanobacterial strains, from both aquatic and terrestrial habitats,[11,12] the best characterised cyanobacterial pilin being that of *Synechocystis* CB3.[13] Despite numerous studies on the occurrence of pili in cyanobacteria, cyanobacterial pili are still poorly characterised.[12] To our knowledge, there are no published data on any sequences of cyanobacterial pilins. In this paper, a partial purification procedure and amino terminal sequencing of *Synechococcus* 6301 pilin are described.

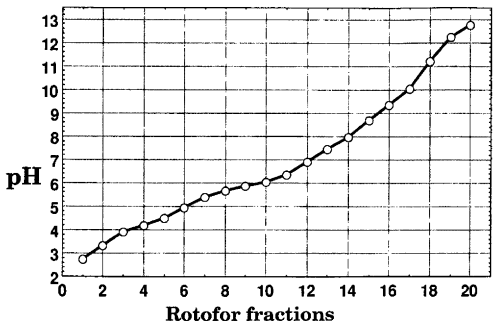

Fig. 1. The pH–gradient profile of the Rotofor fractions after isoelectric focusing (IEF). The data are mean values of four different experiments.

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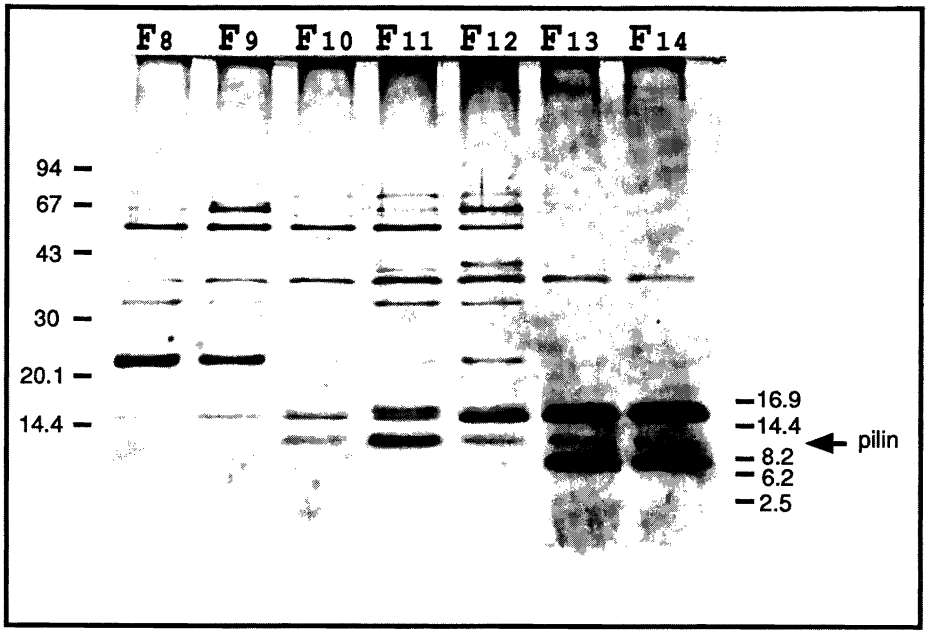

Fig. 2. Analysis of the Rotofor fractions 8–14 (F8–F14) on silver-stained SDS-PAGE gradient gel (12–22%). High and low molecular mass standards are indicated on the left and right, respectively. The arrows indicate the sequenced polypeptides from fractions 13 and 14. The diffusely migrating *Synechococcus* 6301 pilin migrates with a relative molecular mass of 11 kDa.

Experimental

Membrane isolation. Cells of *Synechococcus* 6301 were grown at 30°C in the medium BG-11[14] supplemented with 10 mM TES [2-tris(hydroxymethyl)methylaminoethanesulfonic acid)], pH 8.2. Cultures were stirred continuously, bubbled with 5% CO_2 in N_2 and illuminated with white light (400 μE m^{-2} s^{-1}). Cells in the late logarithmic growth phase were harvested and membranes prepared as described by Harrison *et al.*[15] with some modifications. The cell suspension, including a few milligrams of DNAase and RNAase, was disrupted using a bead beater, 20 cycles of 20 s at 3 min intervals between each cycle. Membranes were collected by ultracentrifugation (100000 g for 40 min at 4°C) with several washings to remove phycobiliproteins. Membranes were finally resuspended to a chlorophyll concentration of 1 mg ml^{-1} in 10% glycerol, 50 mM HEPES–NaOH pH 7.8, 10 mM $MgCl_2$ and either used directly or stored at −80°C for later use.

Solubilization of the membrane proteins and purification of pilin. Membranes (about 150 mg protein equivalent to 5 mg chlorophyll) were pelleted at 100000 g for 40 min at 4°C. The pellet was homogenised and incubated (on ice for 1 h with gentle stirring) in 10 ml of 1 mM HEPES, pH 7.8, containing 5 mM NaF, 5% glycerol, 1% Thesit® (purchased from Boehringer Mannheim), 1% Triton X-100 and 0.5% dodecyl β-D-maltoside, and centrifuged at 150000 g for 40 min. The supernatant, containing solubilised proteins was collected. The solubilised proteins

Table 1. Amino acid terminal sequence analysis of the cyanobacterial *Synechococcus* 6301 pilin.

Cycle	PTH-amino acid residues	Symbol	pmol
1	–	–	–
2	Thr	T	22.5
3	Leu	L	21.8
4	Val	V	23.9
5	Glu	E	23.0
6	Leu	L	–
7	Leu	L	24.4
8	Val	V	22.1
9	Val	V	23.7
10	Ile	I	22.4
11	Ile	I	22.4
12	Ile	I	23.3
13	Val	V	18.6
14	Gly	G	30.4
15	Ile	I	18.6
16	Leu	L	–
17	Ala	A	22.1
18	Ala	A	22.6
19	Val	V	16.6
20	Ala	A	20.6
21	Leu	L	15.2
22	Pro	P	16.0
23	Asn	N	13.1
24	Leu	L	14.5
25	Leu	L	16.4
26	Ala	A	20.7
27	Gln	Q	13.8
28	Thr	T	16.6
29	Asp	D	13.0
30	–	–	–

were loaded on a Bio-Rad Rotofor [preparative isoelectric focusing (IEF)] system with addition of 2 ml ampholyte solution, Bio-Lyte® 3–10 (purchased from Bio-Rad). The Rotofor chamber was filled with 45 ml extraction buffer (excluding NaF) with addition of 1 mM dithiothreitol and 1 mM EDTA (final concentrations).

Focusing was carried out in the Rotofor system for 5 h at 12 W constant power at 4 °C. The initial voltage was 240 V and the current 50 mA. At equilibrium the values were 1020 V and 12 mA, respectively. Twenty fractions were collected and their pH values measured. Aliquots of each fraction were analysed by SDS-PAGE microslab on 12–22% acrylamide gradient gels.[16]

Amino acid terminal sequencing. Purified samples were loaded onto Tris–Tricine system gels containing 12% polyacrylamide and electrophoresed according to Schägger and von Jagow.[17] After electrophoresis, the proteins were transferred onto PVDF (polyvinylidene difluoride) microporous membranes according to Matsudaira's procedure.[18] The PVDF membrane was washed in ultrapure water for 2 h, dried and then stained with 0.05% Sulphorhodamine S in 20% methanol with 0.2% acetic acid. Polypeptide bands on the membrane were sequenced using a gas-phase sequencer (Applied Biosystems model 477A LP).

Results and discussion

To optimise solubilization of proteins associated with membranes extracted from *Synechococcus* 6301, different detergent combinations were applied: Triton X-100, Thesit®, sodium cholate, MEGA-9 (nonanoyl-*N*-methylglucamide) and dodecyl β-D-maltoside. At very low buffer capacity, a combination of Triton X-100, Thesit® and dodecyl β-D-maltoside was sufficient for 80–85% solubilization of membrane proteins (not shown). Fractionation of solubilised membrane proteins was performed using several techniques. Sucrose gradient fractionation and chromatography techniques were applied but these methods were not effective for pilin purification and the yield was very low, presumably because of the hydrophobic nature of the protein (not shown). However, isoelectric focusing using the Rotofor technique looked promising, since the protein mixture remains in the native state in the extraction mixture and can be applied to preparative-scale purifications.

Table 2. Sequence alignment of *Synechococcus* 6301 pilin and various fimbrial proteins derived from pathogenic gram-negative bacterial species. Searches were done on the Swiss–Prot database. ‡ represents regions of identical amino acid sequences. | represents conservative substitutions. Asterisks (*) denote positions of identical matching among all species.

Amino acid terminal sequences	References	Species
** ** * * ***** * *		
TLVELLVVIIIVGILAAVALPNLLAQTD	(this work)	*Synechococcus* 6301
10 20		
‡‡|‡‡||‡‡ ‡‡‡‡‡‡‡‡‡‡		
MNTLQKGFTLIELMIVIAIVGILAAVALPAYQDYTARAQVSEAILLAEGQ	[5]	*Neisseria gonorrhoeae MS11*
MNTLQKGFTLIELMIVIAIVGILAAVALPAYQDYTARAQVSEAILLAEGQ	[19]	*Neisseria gonorrhoeae P9-2*
MNTLQKGFTLIELMIVIAIVGILAAVALPAYQDYTARAQVSEAILLAEGQ	[20]	*Neisseria meningitidis*
10 20 30 40 50		
‡‡|‡‡||‡‡ ‡|‡‡‡‡‡|‡‡‡		
MNAQKGFTLIELMIVIAIIGILAAIALPAYQDYISKSQTTRVVGELAAG	[6]	*Moraxella bovis* (β type)
10 20 30 40 49		
‡‡|‡‡||‡‡ ‡|‡‡‡‡‡|‡‡‡		
FTLIELMIVIAIIGILAAIALPAYQDYISKSQTTRVSGELAAG	[6]	*Moraxella bovis* (α type)
FTLIELMIVIAIIGILAAIALPAYQDYIARAQVSEAFTLADGL	[7]	*Moraxella nonliquefaciens*
10 20 30 40		
‡‡|‡‡||‡| ‡|‡‡‡‡‡ ‡|‡		
MKSLQKGFTLIELMIVVAIIGILAAFAIPAYNDYIARSQAAEGLTLADGL	[8]	*Dichelobacter nodosus 198*
MKSLQKGFTLIELMIVVAIIGILAAFAIPAYNDYIARTQVSEGVSLADGL	[21]	*Dichelobacter nodosus 238*
‡‡|‡‡||‡| ‡|‡‡‡‡‡|‡|‡		
MKAAQKGFTLIELMIVVAIIGILAAIAIPAYQDYTARAQLSERMTLASGL	[22]	*Pseudomonas aeruginosa K122-4*
10 20 30 40 50		
‡‡|‡‡||‡| ‡|‡‡‡‡‡|‡|‡|		
MKAQKGFTLIELMIVVAIIGILAAIAIPQYQDYTARTQVTRAVSEVSAL	[22]	*Pseudomonas aeruginosa P1*
‡‡|‡‡||‡| ‡|‡‡‡‡‡|‡|‡|		
MKAQKGFTLIELMIVVAIIGILAAIAIPQYQDYTARTQVTRAVSEVSAL	[23]	*Pseudomonas aeruginosa 1244*
10 20 30 40 49		

Initial attempts at membrane protein fractionation with the Rotofor cell were less successful owing to low focusing resolution and protein precipitation in the cell. These problems were substantially minimised by readjustment of the extraction buffer content, such as including 5% glycerol in the Rotofor chamber and lowering the ionic strength and buffer capacity. Prefocusing the pH gradient before loading the protein sample also improved separation. However, minor protein precipitation at the Rotofor compartment-ends was observed, but the resolution of the fractionated proteins was unaffected. This effect may be due largely to salting-out of proteins as a result of extreme pH values.

A single Rotofor fractionation of the crude membrane protein extract produced a satisfactory separation of proteins (not shown). The pH gradient formation in the Rotofor chamber (twenty fractions) is continuous and the gradients formed were of approximately 0.2 to 1.4 pH units per fraction (Fig. 1). The cathode and anode side fractions (end compartments) have a steeper gradient due to encroachment of electrolyte, and a higher salt concentrations created during the isoelectrofocusing (IEF) run.

Fig. 2 shows SDS-PAGE analysis of fractions 8–14. Fractions 13 and 14 were most enriched in a diffuse polypeptide which corresponds to pilin. The purification factor of pilin in these two fractions was up to 100-fold. The apparent relative molecular mass of pilin was estimated to be about 11 kDa on SDS-PAGE (Fig. 2). The size of *Synechococcus* 6301 pilin is therefore somewhat smaller than enterobacterial pilin (15–20 kDa),[3–8] but migration on SDS-PAGE may be anomalous because of the hydrophobic nature of the polypeptide.

The 11 kDa pilin from fractions 13 and 14 was amino terminally sequenced up to 28 amino acid residues (Table 1). The first amino acid residue was modified and we were not able to identify it from the standard phenylthiohydantoin (PTH) amino acid analysis. The succeeding amino terminal of 28 amino acids showed a high proportion of very hydrophobic residues (Table 1). Sequence matching and alignments of *Synechococcus* 6301 pilin with related proteins is summarised in Table 2. The amino terminal of the *Synechococcus* 6301 sequence represents mature protein, therefore the initial 6–7 residues (i.e., amino terminal pre-pilin leader sequence) is absent. The initial 6–7 residues (Table 2) of the pilin from other species have been deduced from gene-sequencing.[5–7,19–23] The sequence identities between cyanobacterial pilin and various enterobacterial pilins are *Pseudomonas aeruginosa* (59%), *Neisseria gonorrhoeae* (81%), *Bacteroides nodosus* (62%), *Moraxella bovis* (71%) and *Moraxella nonliquefaciens* (71%). The sequence conservation was remarkably high (60–80%), despite the diversity of bacterial species from which the pilins originated. The amino terminal region of *Synechococcus* 6301 pilin is particularly interesting because of its high conservation and extreme hydrophobicity (Table 2).

Further implications of these results will be explored. In particular, this study represents the first reported sequence of cyanobacterial pilin, and will allow us to identify and clone the pilin structural gene(s). Regulation and control of the *Synechococcus* 6301 gene encoding pilin under environmental conditions could also be investigated. These studies are essential to extend our knowledge of regulation and function of *Synechococcus* 6301 pilin.

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References

1. Sokatch, J. R. In: Gunsalus, I. C., Ed., *The Bacteria*, Vol. 7, Academic Press, New York 1979, p. 229.
2. Woods, D. E., Straus, D. C., Johanson, W. G., Berry, V. K. and Bass, J. A. *Infect. Immun. 29* (1980) 1146.
3. Pasloske, B. L., Finlay, B. B. and Paranchych, W. *FEBS Lett. 183* (1985) 408.
4. Sastry, P. A., Finlay, B. B., Pasloske, B. L., Paranchych, W., Pearlstone, J. R. and Smillie, L. B. *J. Bacteriol. 164* (1985) 571.
5. Meyer, T. F., Billyard, E., Haass, R., Storzbach, S. and So, M. *Proc. Natl. Acad. Sci. USA 81* (1984) 6110.
6. Marrs, C. F., Schoolnik, G., Koomey, J. M., Hardy, J., Rothbard, J. and Falkow, S. *J. Bacteriol. 163* (1985) 132.
7. Frøholm, L. O. and Sletten, K. *FEBS Lett. 73* (1977) 29.
8. Ellemaan, T. C. and Hoyne, P. A. *J. Bacteriol. 160* (1984) 1184.
9. Gaastra, W. and de Graaf, F. K. *Microbiol. Rev. 46* (1982) 129.
10. MacRae, T. H., Dobson, W. J. and McCurdy, H. D. *Can. J. Microbiol. 23* (1977) 1096.
11. Vaara, T. *Can. J. Microbiol. 28* (1982) 929.
12. Vaara, T. and Vaara, M. *Methods Enzymol. 167* (1988) 189 and references cited therein.
13. Vaara, T., Ranta, H., Lounatmaa, K. and Korhonen, T. K. *FEMS Microbiol. Lett. 21* (1984) 329.
14. Rippka, R., Deruelles, J., Waterburg, J. B., Herdman, M. and Stanier, R. Y. *J. Gen. Microbiol. 111* (1979) 1.
15. Harrison, M. A., Tsinoremas, N. F. and Allen, J. F. *FEBS Lett. 282* (1991) 295.
16. Laemmli, U. K. *Nature (London) 227* (1970) 680.
17. Schägger, H. and von Jagow, G. *Anal. Biochem. 166* (1987) 368.
18. Matsudaira, P. *J. Biol. Chem. 262* (1987) 10035.
19. Perry, A. C. F., Nicolson, I. J. and Saunders, J. R. *Gene 60* (1987) 85.
20. Potts, W. J. and Saunders, J. R. *Mol. Microbiol. 2* (1988) 647.
21. Elleman, T. C. and von Ahlefeldt, D. A. *Nucleic Acids Res. 15* (1987) 7189.
22. Pasloske, B. L., Sastry, P. A., Finlay, B. B. and Paranchych, W. *J. Bacteriol. 170* (1988) 3738.
23. Castric, P. A., Sidberry, H. F. and Sadoff, J. C. *Mol. Gen. Genet. 216* (1989) 75.

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