

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

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Nore, B. F., Harrison, M. A., Keen, J. N. and Allen, J. F., 1994. Partial Purification of a Cyanobacterial Membrane Protein with Amino Terminal Sequence Similarity to the *N*-Methylphenylalanine Pilins. – Acta Chem. Scand. 48: 578–581 © Acta Chemica Scandinavica 1994.

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.

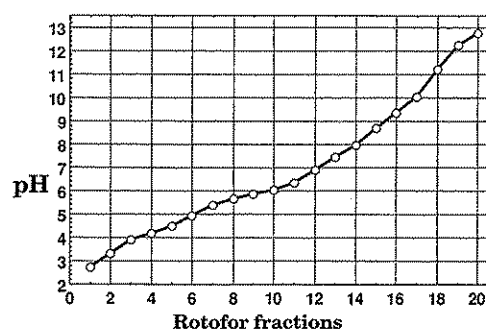


Fig. 1. The pH-gradient profile of the Rotofor fractions after isoelectric focusing (IEF). The data are mean values of four different experiments.

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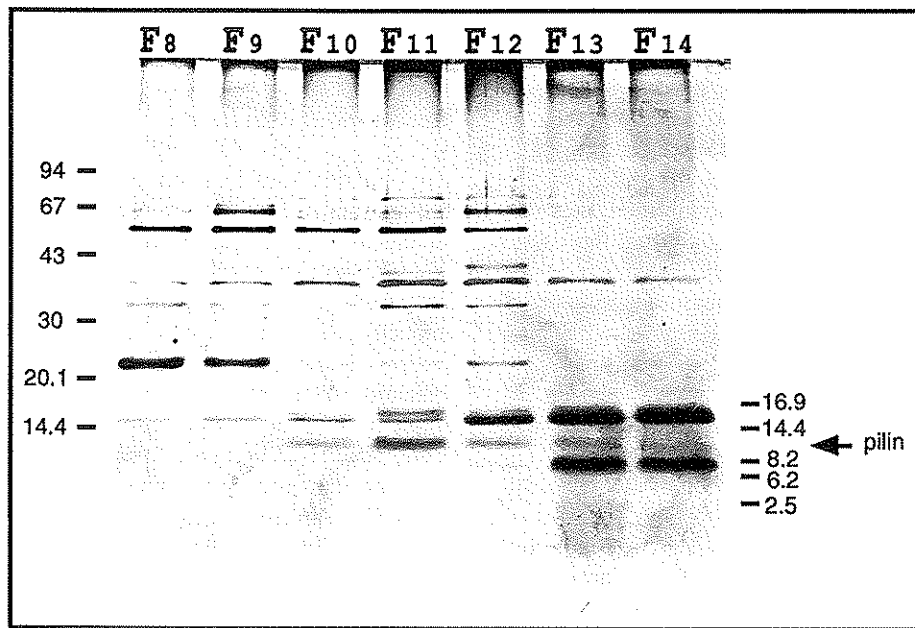


Fig. 2. Analysis of the Rotofor fractions 8–14 (F8–F14) on silver-stained SDS-PAGE gradient gel (12–22%). High and low molecular mass standards are indicated on the left and right, respectively. The arrows indicate the sequenced polypeptides from fractions 13 and 14. The diffusely migrating *Synechococcus* 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-11¹⁴ supplemented with 10 mM TES [2-tris(hydroxymethyl)methylaminoethanesulfonic acid], pH 8.2. Cultures were stirred continuously, bubbled with 5% CO₂ in N₂ 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 (100 000 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 MgCl₂ 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 100 000 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 150 000 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	–	–	–

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.

Acknowledgements. This work was supported by the Nordic Energiforsknings Program (NLVF) and Carl Tryggers Stiftelse För Vetenskaplig Forskning.

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Received January 5, 1994.