the FEBS Journal

Proteomics of *Synechocystis* sp. PCC 6803

Identification of novel integral plasma membrane proteins

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Keywords
cyanobacteria; integral proteins; plasma membrane; proteome; *Synechocystis* 6803

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(Received 8 September 2006, revised 27 November 2006, accepted 5 December 2006)

doi:10.1111/j.1742-4658.2006.05624.x

The cyanobacterial plasma membrane is an essential cell barrier with functions such as the control of taxis, nutrient uptake and secretion. These functions are carried out by integral membrane proteins, which are difficult to identify using standard proteomic methods. In this study, integral proteins were enriched from purified plasma membranes of *Synechocystis* sp. PCC 6803 using urea wash followed by protein resolution in 1D SDS/PAGE. In total, 51 proteins were identified by peptide mass fingerprinting using MALDI-TOF MS. More than half of the proteins were predicted to be integral with 1–12 transmembrane helices. The majority of the proteins had not been identified previously, and include members of metalloproteases, chemotaxis proteins, secretion proteins, as well as type 2 NAD(P)H dehydrogenase and glycosyltransferase. The obtained results serve as a useful reference for further investigations of the address codes for targeting of integral membrane proteins in cyanobacteria.

Cyanobacteria are unique among prokaryotes because of their complex membrane organization. Similar to other Gram-negative bacteria, cyanobacteria have an envelope consisting of the outer and plasma membranes and an intervening peptidoglycan layer. In addition, these organisms have a distinct intracellular membrane system, the thylakoids, which are energy-transducing membranes and the site of both photosynthesis and respiration. The plasma membrane of all cell types contains important proteins/protein complexes involved in different functions, for example, nutrient uptake, efflux or secretory pumps and energy-transducing systems. Because of difficulties purifying cyanobacterial membranes, very few studies on proteomic analysis of plasma membrane proteins have been reported. Pure plasma membranes from *Synechocystis* sp. PCC 6803 (henceforth referred to as *Synechocystis*) were isolated using aqueous two-phase partitioning and used in proteomic studies [1,2]. In total, 79 different proteins were identified in these investigations. However, only 18 of these are integral proteins (known or predicted), and the majority have only one transmembrane helix. Analysis of the *Synechocystis* genome using the TMHMM program (http://www.cbs.dtu.dk/services/TMHMM-2.0/) predicts that $\sim$700 α-helical membrane-spanning proteins are distributed between the plasma and thylakoid membranes. The low number of identified integral membrane proteins are explained by the well-known limitations of using 2D gels (IEF/SDS/PAGE) to isolate hydrophobic proteins [3,4], mainly due to the low solubility of hydrophobic proteins in urea/dithiothreitol, and to aggregation during IEF. In this study we used 1D SDS/PAGE to avoid this problem. In order to enrich the integral membrane proteins and obtain better resolution, membranes were washed with urea. Proteins were identified

Abbreviations
ABC, ATP-binding cassette; ER, endoplasmic reticulum; MCP, methyl-accepting chemotaxis proteins; PMF, peptide mass fingerprinting; PS, photosystem; Sec, general secretion pathway; Tat, twin-arginine translocation.

by peptide mass fingerprinting (PMF) using MALDI TOF MS coupled to database searching by MASCOT (http://www.matrix.science.com). One protein was identified after peptide sequence analysis using post-source decay with MALDI MS [5].

Results and Discussion

General characteristics of identified plasma membrane proteins from 1D gels

We used 1D SDS/PAGE to separate plasma membrane proteins from *Synechocystis*. To improve the resolution and enrich the integral membrane proteins, membranes were washed with urea. Urea-washed membranes from different preparations gave similar band patterns and, after MALDI-TOF MS analysis, the same proteins (but with varying Mowse scores) were identified from three different gels. Figure 1 shows the typical protein pattern of plasma membranes before urea treatment (lane 2), integral proteins recovered in the pellet (lane 3) and soluble proteins in supernatant (lane 4) for Coomassie Brilliant Blue-stained SDS-polyacrylamide gels (10–18% gradient). The numbers refer to identified proteins listed in Table 1.

Fifty-one different proteins were identified (Table 1) using PMF and MALDI-TOF MS techniques coupled to database search using the MASCOT program. Figure 2 shows a representative spectrum of one of the identified proteins, Slr1512, which is the sodium-dependant bicarbonate transporter SbtA with eight transmembrane helices. The Mowse scores documented in Table 1 are the highest found in any of the analysed gels for every specific protein. One of the main problems in studying membrane proteins is that transmembrane domains usually do not have charged arginine or lysine residues, which are recognized by the protease trypsin because these segments occupy the hydrophobic interior of the lipid bilayer. The foregoing statements, combined with the fact that hydrophobic segments are less easily ionized, account for the poor sequence coverage by MS when dealing with membrane proteins. Despite these difficulties it was found that 26 of the identified proteins were assigned as integral membrane proteins (denoted by b in Table 1) using the TMHMM program [6], and some of these have up to 12 known/predicted transmembrane helices (Table 2), although $\sim$60% have only one or two. The majority of the integral membrane proteins, 19, have not been identified in previous proteomic studies (a in Tables 1 and 2). When analyzing the localization of the matched peptides (Table 2) it was found that, of

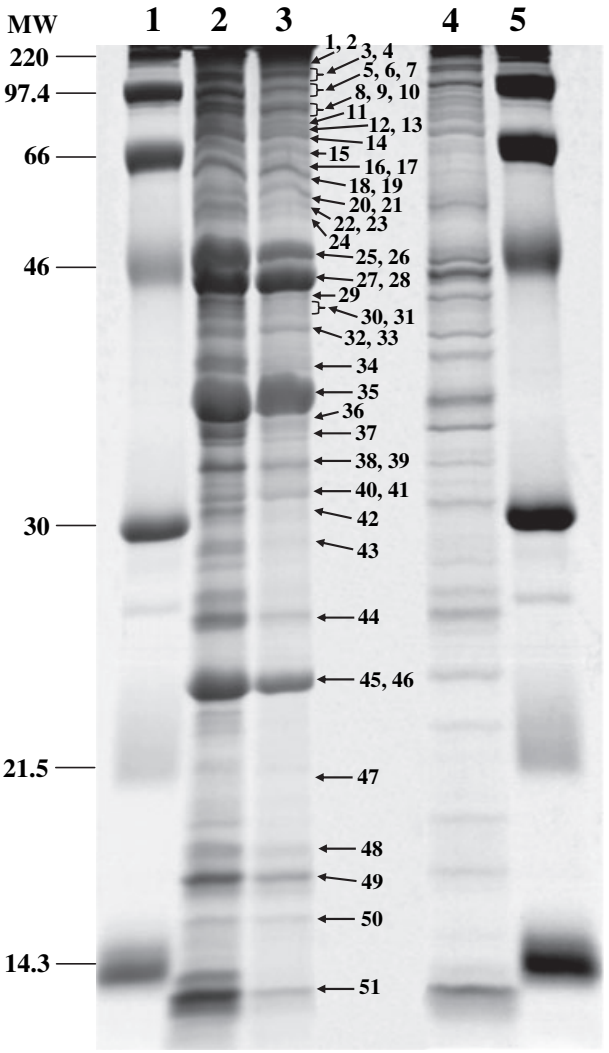

Fig. 1. Coomassie Brilliant Blue-stained 1D gradient (10–18%) gels of plasma membrane proteins of *Synechocystis*. Lanes 1 and 5, molecular mass marker; lane 2, total plasma membrane proteins; lane 3, plasma membrane after urea wash; lane 4, supernatant after urea wash.

the 26 integral membrane proteins identified, 23 had all their matched peptides in the peripheral part of the protein, i.e. the loops or the N– and C-terminals. This finding can be considered a strong indicator of correct identification, particularly for proteins with many transmembrane helices. In some gel bands two or three different proteins were identified. Each protein had enough matching peptides to get a significant score, although the highest scores for each protein (Table 1) may originate from different gels.

Protein 12 was identified as Sll1665 from fingerprinting although with a very low score of 57 (Table 1). Using postsource decay analysis with MALDI MS [5]

Table 1. Proteins identified in the plasma membrane of *Synechocystis*.

Protein No.	ORF	Gene product	Mowse score	Matched peptides/ Total	Cov. %	*M*, kDa theor./exp.	Predicted p*I*
1	slr0369[a,b]	Cation/multidrug efflux system protein	91	10/18	13	117.5/130	4.9
2	slr2131[a,b]	Cation/multidrug efflux system protein	78	9/17	11	115/130	5.0
3	sll0041[a,b]	Methyl-accepting chemotaxis protein, pixJ1	174	17/28	25	97/110	4.8
4	slr1044[a,b]	Methyl-accepting chemotaxis protein, pilJ	93	13/37	14	93.2/110	4.4
5	sll1294[a,b]	Methyl-accepting chemotaxis protein	148	18/38	23	103.1/103	4.6
6	sll1180[a,b]	Toxin secretion ABC transporter ATP-binding protein, HlyB	88	14/40	15	112.4/103	5.7
7	slr0335	Phycobilisome LCM-core membrane linker polypeptide, ApcE	213	22/34	27	100.4/103	9.2
8	slr6071[a,b]	Hypothetical protein	93	13/31	20	84.1/86	5.8
9	sll0923[a,b]	Exopolysaccharide export protein, EpsB	108	12/29	24	83.6/86	5.0
10	slr0798[a,b]	Zinc-transporting P-type ATPase (zinc efflux pump), ZiaA	76	9/24	14	77.1/86	6.0
11	sll1021[b]	Hypothetical protein	118	12/25	21	74.5/80	5.1
12	sll1665[a,b]	Hypothetical protein (*Synechocystis* only)	57	5/11	9	63.5/76	3.5
13	slr1604[a,b,c]	Protease, FtsH4	156	17/44	36	67.3/76	5.2
14	sll1031	Carbon dioxide concentrating mechanism protein, CcmM	80	8/17	15	73.6/72	8.6
15	slr0963	Ferredoxin sulfite reductase	87	9/14	12	71.8/70	8.5
16	slr1609[a]	Long-chain-fatty-acid CoA ligase, FadD	208	21/37	33	77.9/68	6.7
17	slr1390[a,b,c]	Protease, FtsH2	146	19/61	32	72.2/67	5.8
18	slr2105[a,b]	Hypothetical protein	87	10/24	21	65.3/65	4.8
19	slr0765[a,b,c]	Mechanosensitive ion channel, MscS	169	15/26	26	64.5/65	6.7
20	sll1178[a]	Nodulation protein, probable carbamoyl transferase	103	8/11	16	69.5/59	5.7
21	slr1841[c]	Putative porin	116	10/17	18	67.7/59	4.6
22	sll0180[d]	Membrane fusion protein	129	10/16	27	53.9/54	5.8
23	slr1721[a,b]	Hypothetical protein	76	6/10	14	54.5/54	5.4
24	sll1484[a,b]	NADH-dehydrogenase type II, NdbC	94	9/21	22	57.1/52	6.7
25	slr0009	Ribulose bisphosphate carboxylase large subunit, RbcL	140	13/25	31	53/48	6.1
26	slr1908[c]	Putative porin	108	13/34	26	64.5/48	5.2
27	slr0447[d]	Periplasmic-binding protein of the ABC-type, high-affinity urea permease, UrtA	114	9/17	28	48.5/45	4.9
28	slr0040[d]	Bicarbonate transporter, CmpA	128	10/17	29	49.5/45	5.8
29	sll1450[d]	Nitrate transport 45 kDa, NrtA	174	14/26	46	49.1/44	5.3
30	sll0752	Hypothetical protein	88	7/20	39	31.4/43	4.9
31	slr0394	Phosphoglycerate kinase, PgK	76	7/20	25	42/42	5.0
32	slr1295[d]	Periplasmic-binding protein of the ABC-type, iron transport protein, FutA1/SufA	173	13/25	47	39.4/41	4.9
33	slr1128	Hypothetical protein	132	11/23	35	35.7/41	5.7
34	slr0151	Hypothetical protein	83	6/12	27	35.0/39	5.0
35	slr1512[b]	Sodium-dependent bicarbonate transporter, SbtA	90	9/24	14	39.6/37	5.4
36	slr1943[a,b]	Putative glycosyltransferase	86	8/19	22	37.7/36	8.3
37	sll0034[b]	Putative carboxypeptidase, VanY	106	8/19	35	28.6/34	6.1
38	slr0848	Hypothetical protein	108	9/24	35	31.9/33	4.9
39	slr1319[d]	Iron(III) dicitrate transport system permease protein, FecB	107	8/17	36	34.9/33	5.0
40	sll1580	Phycocyanin ass. linker protein, CpcC2	152	10/12	32	32.5/32	9.3
41	sll1579	Phycocyanin, CpcC	134	8/10	31	30.7/32	9.4

Table 1. (Continued).

Protein No.	ORF	Gene product	Mowse score	Matched peptides/ Total	Cov. %	*M*, kDa theor./exp.	Predicted p*I*
42	sll1757[a,b]	Hypothetical protein	77	5/8	15	31.8/30	5.4
43	sll1471	Phycobilisome rod-core linker polypeptide, CpcG	84	8/22	27	28.6/29	9.1
44	slr0677[b]	Biopolymer transport protein, ExbB/TolQ	79	5/8	14	25.0/26	5.2
45	sll1694[b]	Pilin, PilA1	76	5/6	22	17.7/23	4.8
46	sll1404[a,b]	Biopolymer transport protein, ExbB/TolQ	126	7/9	36	23/23	9.1
47	slr0013[b]	Hypothetical protein	137	11/23	66	18.6/20	9.0
48	sll1577	Phycocyanin b subunit, CpcB	98	7/21	51	18.3/18	5.1
49	sll0813[b,c]	Cytochrome *c* oxidase subunit II, CtaC	77	6/14	19	33.5/17	7.6
50	slr0516[a,d]	Hypothetical protein	77	5/12	28	18/16	4.6
51	slr1513	Cyanobacterial hypothetical	85	6/12	38	12.1/13	7.0

[a] Newly identified proteins. [b] Integral membrane protein predicted using TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/). [c] Signal peptides were predicted using SIGNALP 3.0 (http://www.cbs.dtu.dk/services/SignalP/). [d] Lipoproteins were predicted using LIPOP 1.0 (http://www.cbs.dtu.dk/services/LipoP/).

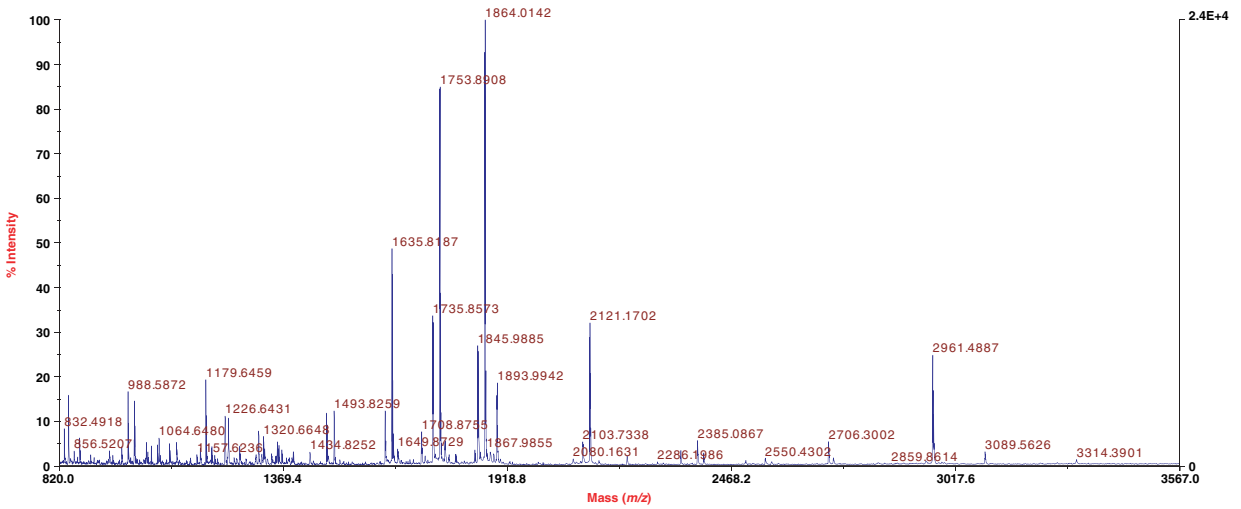

Fig. 2. MALDI-TOF MS spectrum of the peptides generated by trypsin digestion of protein Slr1512, the sodium-dependant bicarbonate transporter SbtA.

a peptide was sequenced (TALEDELQSLR) and the identity (ion score 46) of the protein could be assigned to Sll1665, demonstrating that the fingerprint analysis was correct despite the very low score.

It is well known that most bacterial integral membrane proteins consisting of an α-helix transmembrane structure do not have a cleavable N-terminal signal peptide [7]. This is shown also for the α-helical integral membrane proteins in the plasma membrane of *Synechocystis*. Only 4 of the 26 integral membrane proteins had an N-terminal signal peptide, as predicted by the SIGNALP 3.0 program [8] (Table 2). Two putative porins of β-barrel structure (slr1841 and slr1908) can, as reported previously [1], be found in the plasma membrane on their way to their final localization in the outer membrane. The β-barrel proteins were found to have a predicted general secretion pathway (Sec) N-terminal signal (not shown).

Two of the identified proteins (Sll0923 and Sll1484) have a single transmembrane helix in the C-terminus

Table 2. Integral proteins identified in the plasma membrane of *Synechocystis*.

Protein No.	ORF	Gene product	Signal peptide[b]	No. and position of transmembrane helices[c]	Matched peptides[d] peripheral/total
9	sll0923[a]	Exopolysaccharide export protein, EpsB		1: 716–738	12/12
24	sll1484[a]	NADH-dehydrogenase type II, NdbC		1: 1450–467	7/9
37	sll0034	Putative carboxypeptidase, VanY		1: 40–59	8/8
23	slr1721[a]	Hypothetical protein		1: 21–38	6/6
12	sll1665[a]	Hypothetical protein (*Synechocystis* only)		1: 5–27	5/5
17	slr1390[a]	Protease, FtsH1	17	1: 118–140	18/19
13	slr1604[a]	Protease, FtsH3	25	1: 82–104	17/17
8	slr6071[a]	Hypothetical protein		1: 12–31	13/13
47	slr0013	Hypothetical protein		1: 13–35	11/11
11	sll1021	Hypothetical protein		1: 60–82	12/12
45	sll1694	Pilin, PilA1		1: 20–42	5/5
3	sll0041[a]	Methyl-accepting chemotaxis protein, pixJ1		2: 201–223, 247–266	14/17
4	slr1044[a]	Methyl-accepting chemotaxis protein, pilJ		2: 382–404, 447–466	13/13
5	sll1294[a]	Methyl-accepting chemotaxis protein		2: 220–242, 528–550	18/18
36	slr1943[a]	Glycosyltransferase		2: 247–269, 284–306	8/8
49	sll0813	Cytochrome c oxidase subunit II, CtaC	27	2: 20–42, 62–84	6/6
46	sll1404[a]	Biopolymer transport protein, ExbB/TolQ		3: 108–130, 135–157, 150–172	7/7
44	slr0677	Biopolymer transport protein, ExbB/TolQ		3: 13–35, 111–133, 153–175	5/5
42	sll1757[a]	Hypothetical protein		3: 29–51, 66–88, 109–131	5/5
18	slr2105[a]	Hypothetical protein		4: 13–35, 39–58, 78–100, 570–592	10/10
6	sll1180[a]	Toxin secretion ABC transporter ATP-binding protein, HlyB		4: 450–472, 492–514, 562–584, 591–613	14/14
10	slr0798[a]	Zinc-transporting P-type ATPase (zinc efflux pump), ZiaA		5: 111–128, 138–156, 338–360, 375–397, 677–699	9/9
19	slr0765[a]	Mechanosensitive ion channel, MscS	37	5: 160–182, 250–272, 277–299, 345–367, 371–393	15/15
35	slr1512	Sodium-dependent bicarbonate transporter, SbtA		8: 15–37, 42–61, 71–93, 100–122, 137–159, 279–301, 305–322, 343–365	7/9
1	slr0369[a]	Cation/multidrug efflux system protein		11: 297–316, 323–345, 350–372, 393–415, 425–447, 488–510, 825–847, 854–873, 893–915, 941–963, 973–995	10/10
2	slr2131[a]	Cation/multidrug efflux system protein		12: 12–34, 342–361, 368–385, 395–415, 440–462, 477–499, 534–556, 872–891, 898–917, 927–949, 970–989, 1004–1026	9/9

[a] Newly identified proteins. [b] Position of cleavage site predicted using SIGNALP 3.0 (http://www.cbs.dtu.dk/services/SignalP/). [c] Number and position of transmembrane helices predicted using TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/). [d] Matched peptides in the peripheral part of the protein/total matched peptides.

(Table 2) and a large hydrophilic N-terminal domain with no predicted signal peptide. In eukaryotic cells, these types of integral membrane protein are called tail-anchored proteins and their hydrophilic N-terminal domain is described as being cotranslationally folded before the hydrophobic tail emerges from the ribosome [9]. The mechanism of insertion into the endoplasmic reticulum (ER) membrane or outer mitochondrial membrane, the two known locations [10] for tail-anchored proteins, is not known, but is suggested to be Sec independent [9]. Sorting for either the ER or the outer mitochondrial membrane is dependent on the presence/absence of positively charged amino acids directly after the C-terminal transmembrane segment [11]. To date, no tail-anchored proteins have been studied in bacteria. The two tail-anchored proteins found in this study both have positively charged amino acids at this position (not shown). No tail-anchored protein has, however, been identified in the thylakoid membrane of *Synechocystis*, so the significance of this remains to be investigated.

Peripheral proteins on the periplasmic side of the plasma membrane should possess a cleavable N-terminal sequence of the Sec, Tat or lipoprotein type for targeting to the membrane. In previous proteomic work on the plasma membranes of *Synechocystis* [1,2] it was found that of the peripheral proteins the Sec and lipoprotein types constituted 45% each, whereas only 10% were Tat proteins. Because in this study the membranes were washed with urea to remove the peripheral proteins for enrichment of the integral membrane proteins no peripheral protein with a Sec or Tat signal was found. Seven proteins with a lipoprotein motif (d in Table 1), as predicted using LIPOP 1.0 (http://www.cbs.dtu.dk/services/LipoP) [12], were identified. One of the lipoproteins (Slr0516) was not detected in previous studies [1,2]. For four of the predicted lipoproteins the N-terminal had an RRXΦΦ-motif (Φ, representing a hydrophobic amino acid residue) typical for proteins translocated by the Tat-translocase [13]. The TATP program [14] did not recognize these signals. It is not known, however, if lipoproteins can be translocated via the Tat-system in Gram-negative bacteria. In a Gram-positive bacteria, *Streptomyces coelicolor* A3, two protein constructs were made consisting of endogenous lipoprotein signal sequences, containing the twin-arginine motif which were fused with a reporter protein. Both fusion proteins were shown to be translocated via the Tat-translocase [15].

Sixteen soluble proteins with no predicted signal peptide were also present in the plasma membrane preparation (Table 1). Because of the abundance of the phycobilisome complex and carboxysomes, five different subunits of the phycobilisome complex were found associated with the plasma membrane, as well as the large subunit of Rubisco and the CcmM subunit of the carboxysome. Furthermore, five hypothetical proteins, a nodulation protein, long-chain fatty-acid CoA ligase, phosphoglycerate kinase and ferredoxin sulfite reductase were associated with the plasma membrane. Ferredoxin sulfite reductase has previously been shown to be associated with the total membrane fraction [16]. The rest of these proteins are either true peripheral proteins on the cytoplasmic side of the membrane or abundant cytosolic proteins coincidently associated with the membrane. However, only two hypothetical proteins and phosphoglycerate kinase have been identified in previous proteomic studies of the total soluble protein fraction from *Synechocystis* [17–20], and therefore only these can be considered as abundant cytosolic proteins and the rest as peripheral plasma membrane proteins.

p*I* values correlated with protein subcellular localization

For six bacteria/archaea with sequenced genomes, including *Synechocystis*, estimated p*I* values of all predicted proteins were shown to have a bimodal distribution for each species [21] with one peak centred at ~p*I* 5 and the other at ~p*I* 9. In the same investigation, the analyses were repeated using two subsets of proteins from the SWISS-PROT database with the annotation 'Subcellular location: cytoplasmic' and 'Subcellular localization: integral membrane proteins'. It was shown that cytoplasmic proteins exhibited a distinct clustering around p*I* 5–6, whereas integral membrane proteins were clustered primarily around p*I* 8.5–9. We analysed the *Synechocystis* genome, based on all 3168 ORFs, using TMHMM [6] to predict integral proteins with transmembrane helices. In order to exclude the hydrophobic part of the putative N-terminal signal sequences, the analysis was carried out in combination with the SIGNALP program [8]. It was found that ~700 of the *Synechocystis* proteins have 1–17 transmembrane helices, and ~30% of these have one transmembrane helix. Furthermore, the integral *Synechocystis* membrane proteins were shown to have a bimodal p*I* profile with an equal distribution between low and high p*I* values (Fig. 3A), which contradicts the results of Schwartz *et al.* [21], described above, for integral membrane proteins derived from the SWISS-PROT database based on annotation. However, when comparing p*I* values for proteins with one or more transmembrane helices a difference was seen. Proteins with

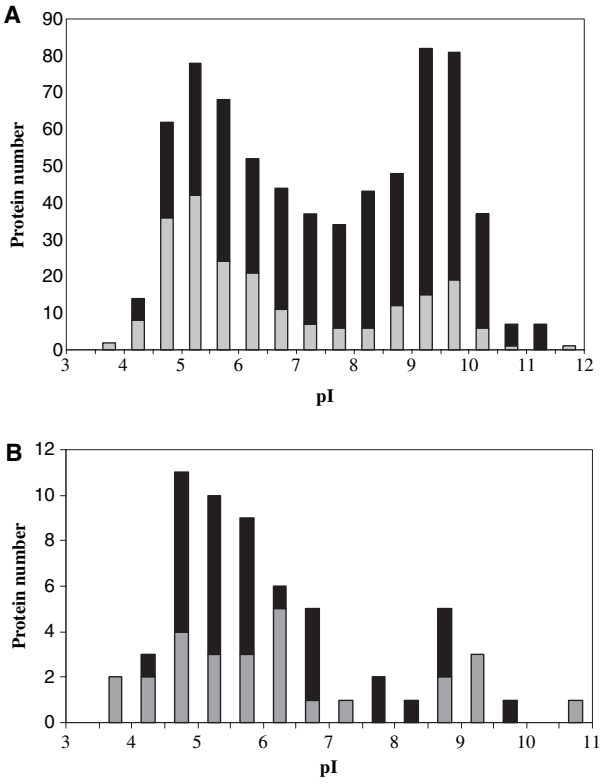

Fig. 3. p*I* values at 0.5 unit intervals for integral membrane proteins in *Synechocystis*. (Lower) Proteins with 1 predicted transmembrane helix. (Upper) Proteins with 2–17 predicted transmembrane helices. (A) All predicted integral membrane proteins. (B) Experimentally identified integral membrane proteins [22,24].

one transmembrane helix have mostly low p*I* values (Fig. 3A, lower part of bars), a property shared with the soluble proteins, whereas those with more transmembrane helices (upper part of bars) have higher p*I* values.

Proteomic studies using blue native gels have identified integral membrane proteins as part of the two photosystems [22] and NADH dehydrogenase complex [23]. In addition, integral membrane proteins have been identified in 1D gels of isolated photosystem (PS) I and II complexes [24,25] and purified thylakoid membrane preparations [26]. In total, 60 different integral membrane proteins were experimentally identified in these studies and the p*I* distribution is shown in Fig. 3B. Although analysis of the total integral membrane proteome (Fig. 3A) showed an equal distribution between low and high p*I* values, the experimentally identified proteins were mostly found to have low p*I* values. The reason for this discrepancy is not clear because in 1D gels and blue native gels proteins with high p*I* values should be possible to resolve.

Proteases in *Synechocystis*

The *Synechocystis* genome contains a number of genes that encode different proteases [27]. In previous proteomic studies we identified two members of the Deg protease family: DegP/HtrA (Slr1204) in the outer membrane [28] and DegQ/HhoA (Sll1679) in the plasma membrane and the periplasmic fraction [2,29]. The genome contains three predicted C-terminal protease (ctp) genes, and all are shown to be expressed. We have shown that CtpA (Slr0008) is present in the plasma membrane [30], CtpB (Slr0257) in the periplasm [29] and CtpC (Slr1751) in all compartments investigated: periplasm [29], plasma [1], outer [28] and thylakoid membranes [26]. Of the eight genes encoding members of the Clp family in *Synechocystis* only ClpC (*sll0020*) has been shown to be expressed. We have shown that ClpC is associated with both thylakoid [26] and plasma membranes [2]. Furthermore, two soluble processing metalloproteases PqqE (Sll0915) and YmxG (Slr1331) are present in the periplasm [29]. A putative carboxy peptidase (Sll0034) anchored to the plasma membrane with one transmembrane helix, and an active site in the periplasm has been identified previously [1] and was also found in this study (Tables 1,2).

FtsH, an ATP-dependent zinc metalloprotease, was initially discovered in an *Escherichia coli* cell-division mutant and was found to be a member of the AAA family of ATPases [31]. All prokaryotic genomes contain a single FtsH gene. The only exception is the cyanobacteria, which contain four FtsH genes [32]. A plant homologue of the bacterial FtsH protease was first identified as a chloroplast protein integral to the thylakoid membrane [33] and was later shown to be involved in the light-induced degradation of the PS II D1 protein [34]. We now know that plant FtsH proteases constitute a multigene family and in *Arabidopsis* at least nine members are present in chloroplasts [35]. Genome analysis of the green algae *Chlamydomonas reinhardtii* (JGI CHLAMY v3.0; http://genome.jgi-psf.org/Chlre3/Chlre3.info.html) also reveals nine FtsHs with amino acid sequences highly similar to the four cyanobacterial enzymes (*e*-values between −104 and 0.0). Thus it appears that multiplication of FtsH genes correlates with the evolution of oxygenic photosynthesis. In this study we show that two of the four FtsH gene products of *Synechocystis* are localized in the plasma membrane: FtsH1 (Slr1390) and FtsH3 (Slr1604). FtsH4 (Sll1463) was previously found in the thylakoid membrane [26] as well as FtsH2 (Slr0228) (B. Norling *et al.*, unpublished results). Both these FtsHs in the thylakoid membrane, as well as the plasma membrane enzymes (Table 2), are integral membrane proteins.

Interestingly, the two plasma-membrane proteases (FtsH1 and FtsH3) are essential for cell viability and growth [23]. Mutation of FtsH2 is shown to affect PS I activity, indicating the involvement of this protease in biogenesis [32]. Recent studies show that FtsH2 plays an important role in the photoprotection of PS II, involved in early steps of D1 degradation [36,37]. Disruption of FtsH4 has no obvious phenotype [32].

Homologous methyl-accepting chemotaxis proteins (MCP) in *Synechocystis*

The methyl-accepting chemotaxis proteins (MCP)/CheA/CheY system is the major regulatory pathway of signal transduction for bacterial chemotaxis/phototaxis. In the *Synechocystis* genome there are three sets of MCP/CheA/CheY systems [38,39]. In this study, all three MCP homologues were found in the plasma membrane (Sll0041, Slr1044 and Sll1294).

*Sll*0041 is part of the gene cluster *pix*GHIJ1J2L (positive phototaxis) and is predicted to encode PixJ1, a phytochrome-like photoreceptor that is essential for positive phototaxis. PixJ1 possess two GAF domains, which are known to be present in phytochromes and cGMP-specific phosphodiesterases. Mutagenesis shows that the second domain is responsible for chromophore binding [39–41].

*Slr*1044 is part of the gene cluster *pil*GHIJ, encoding PilJ, which is required for pilus assembly, motility and natural transformation competency with extrageneous DNA. Disruption of *pilJ* leads to loss of motility due to a dramatically reduced number of thick pili. Moreover pilJ mutant retains very low competency in DNA uptake [42].

*Sll*1294 is part of gene cluster *sll*1291/*sll*1292/*sll*1293/*sll*1294/*sll*1295. The only mutagenic experiment performed show that disruption of none of these genes affected phototactic motility [42]. Although it is now shown that the *sll*1294 gene is expressed, the function of this protein or this third MCP/CheA/CheY system remains to be elucidated.

ExbB/TolQ proteins

In *E. coli* and related Gram-negative bacteria, two systems (TonB–ExbB–ExbD and TolA–TolQ–TolR) are able to transmit electrochemical potential across the cytoplasmic membrane to outer membrane receptors and channels and therefore energize active transport across the latter [43,44]. It has been shown that the integral plasma membrane proteins TonB, ExbB and ExbD are homologous to TolA, TolQ and TolR, respectively. Moreover ExbB/TolQ and ExbD/TolR share the same membrane topology [43].

The *Synechocystis* genome contains two ExbB/TolQ (*slr*0677 and *sll*1404) and two ExbD/TolR (*slr*0678 and *sll*1405) homologues, but no TonB/TolA homologue. *Synechocystis* genes are organized in two operons, one is *sll*1404/*sll*1405/*sll*1406, where *sll*1406 encodes the outer membrane receptor FhuA, and the other is *slr*0677/*slr*0678, encoding ExbB/TolQ and ExbD/TolR. In previous studies we identified Slr0677 and Sll1405 in the plasma membrane [1], and Sll1406 in the outer membrane [28]. In this study the last protein (Sll1404) from the *sll*1404–*sll*1406 operon was identified.

Type I secretion and multidrug efflux pumps

In Gram-negative bacteria there are two export systems for different compounds such as drugs, toxins, sugars, ions, proteins and more complex organic molecules. Both have a tripartite structure consisting of a plasma membrane translocase, membrane fusion or adaptor proteins and a specific outer membrane protein, TolC [45]. The three parts form a contiguous protein complex spanning the bacterial cell envelope allowing secretion of substances without stable periplasmic intermediates. In the type I secretion pathway the plasma membrane translocase is an ATP-binding cassette (ABC) transporter with energy provided via ATP hydrolysis, whereas drug efflux occurs via a plasma membrane proton antiporter [46]. *E. coli* prototypes of these two export systems are the HlyBD/TolC haemolysin secretion system [47] and the ArcAB/TolC drug-efflux pump [48], respectively.

In this study we identified the plasma membrane translocase, HlyB (Sll1180), of the haemolysin secretion system. The membrane fusion protein, HlyD (Sll1181), and the outer membrane protein, TolC (Slr1270), have been identified in a previous proteomic study [28].

Slr0369 and Slr2131, identified in this study, are homologous to the proteins of the ArcB/ArcD/ArcF family, which constitute the plasma membrane component of cation/multidrug efflux pumps. Both proteins belong to the RND (resistance nodulation cell division) family and the two are highly homologous to each other (E-value = 0). In the *Synechocystis* genome three more genes coding for proteins of this family are present. The structure of the major multidrug exporter ArcB in *E. coli* has been determined previously [49]. The *Synechocystis* homologues Slr0369 and Slr2131 are the two largest proteins identified in this study (Table 1) with molecular masses around 120 kDa and

11/12 predicted transmembrane helices, respectively. Slr2131 has two large periplasmic domains from which six and five peptides were identified as well as the N-terminal peptide. Slr0369 has a large hydrophilic N-terminal domain from which five peptides were identified and the remaining five peptides identified came from the only large loop region between transmembrane helices six and seven.

NAD(P)H dehydrogenases

Membrane-bound bacterial pyridine nucleotide dehydrogenases can be divided into two groups called type 1 and type 2 NAD(P)H dehydrogenases, NDH-1 and NDH-2 [50]. Mitochondrial NADH type I is a multi-subunit complex that has recently been analysed using 2D blue native/SDS/PAGE in thylakoid membranes from *Synechocystis* [22,51]. NDH-2 enzymes, by contrast, are single polypeptides. Three putative genes for NDH-2 proteins (*slr*0851, *slr*1743 and *sll*1484) are found in the *Synechocystis* genome, and all three gene products contain the NAD(P)H and flavin adenine-binding motifs [52]. From mutagenic studies it is concluded that NDH-2s do not have a significant catalytic role in respiration, but may serve as redox sensors in the membrane (PQ pool) and/or the NADH/NAD ratio. NDH-2 was therefore suggested to be localized in the thylakoid membrane. In this study we show that one of these NDH-2s, Sll1484, with one predicted transmembrane helix, is present in the plasma membrane (Tables 1,2).

Glycosyltransferases

Glycosyltransferases constitute one of the largest groups of enzymes and are usually classified, on the basis of sequence comparisons, into many families of varying similarity using the CAZY systematic sequence database (http://afmb.cnrs-mrs.fr/CAZY/index.html). These enzymes catalyse the transfer of sugar moieties from activated donor molecules, such as UDP-glucose and GDP-mannose, to specific acceptors including cellulose and dolichol phosphate. *Synechocystis*, and several other cyanobacteria, contain the largest number of predicted glycosyltranferases in relation to genome size. Among 61 predicted glycosyltransferases in *Synechocystis,* Slr1943, is the first to be identified at the protein level. It contains two predicted C-terminal transmembrane helices (Table 2). A BLAST similarity search revealed that many cyanobacterial genomes contain two genes with high similarity in both membrane topology and sequence. Most glycosyltransferases are not predicted to be integral membrane proteins. However, the closest *E. coli* homologues to Slr1943 are two putative glycosyltransferases (*gi*16130283, *gi*16030189) with the same membrane topology. The specific catalytic functions of these membrane bound enzymes remain unknown.

Hypothetical proteins

In previous proteomic studies on the periplasmic fraction [29], plasma [1,2], outer [28] and thylakoid [26] membranes, $\sim$30% of the identified proteins were hypothetical with no known function. In this study $\sim$30% of the identified proteins are hypothetical proteins and more than half are newly identified (Table 1). Among the new hypothetical proteins one is a soluble protein with a pentapeptide repeat (Slr0516) and the remaining six, are integral membrane proteins with one to four predicted transmembrane helices (Table 2). Slr6071 is coded by the pSYSX plasmid, one of four large plasmids in *Synechocystis* [53]. Slr2105 with five predicted transmembrane helices contains a GldG domain, an auxiliary component of an ABC-type transport system involved in gliding motility [54]. Sll1757 and Slr1721 are hypothetical proteins, the genes of which are only found in cyanobacterial genomes and the gene encoding Sll1665 is only present in *Synechocystis*.

Miscellaneous proteins

It is known that mechanosensitive ion channels play an important role in transducing physical stresses at the cell membrane into an electrochemical response providing cell protection [55]. In the *Synechocystis* genome there are nine genes encoding putative mechanosensitive ion channels [56]. One, *slr0875*, which belongs to the protein family of MscL, mechanosensitive channel with large conductance, has been shown to code for a protein involved in Ca^{2+} release induced by plasma membrane depolarization under temperature stress [57]. The other eight predicted mechanosensitive ion channels belong to the MscS family with small conductance. We have identified the first cyanobacterial MscS family member, Slr0765. The structure of the *E. coli* homologue protein, YggB, is resolved [58]. YggB folds as a membrane-spanning homo-heptamer with large N- and C-terminal cytoplasmic regions. The predicted monomer membrane topology for Slr0765 is similar to the established structure of YggB, although the *Synechocystis* MscS monomer possesses five transmembrane helices (Table 2) compared with three in YggB. In addition, Slr0765 has a predicted signal peptide.

The gene *slr*0798, annotated *zia*A, encodes a polypeptide with sequence features of heavy metal transporting P-type ATPase, showing five predicted transmembrane helices and including a soluble N-terminal metal-binding domain [59]. Disruption of *zia*A in *Synechocystis* leads to reduction of Zn tolerance. The suggested localization of ZiaA in the plasma membrane of *Synechocystis* is verified in this study (Tables 1,2).

Concluding remarks

Very few studies on integral membrane proteins from the plasma membrane of cyanobacteria have been carried out. We focused on the identification of integral membrane proteins in the plasma membrane of *Synechocystis* sp. PCC 6803. The proteins were separated on 1D SDS/PAGE, digested with trypsin and identified using MALDI-TOF MS analysis combined with a database search. Enrichment of integral membrane proteins from purified plasma membrane allowed the identification of 26 proteins containing 1–12 predicted transmembrane helices. Of these, 19 had not been identified previously at the protein level. In total, 51 different proteins were identified. Similar to previous subproteomic studies [2,26,28,29,60], $\sim$30% of the identified proteins were hypothetical proteins of unknown function. Peptide mass fingerprinting using MALDI-TOF analyses of peptides is suitable for the rapid identification of proteins from organisms with known genomes. However, due to the nature of integral membrane proteins, with most of the arginines and lysines usually confined in the loops between transmembrane helices, it is difficult to obtain peptides with masses suitable for peptide mass fingerprinting analysis. In addition it is difficult to detect hydrophobic peptides due to inherently low gas phase basicity and analyte suppression by highly hydrophobic peptides. Despite this, 25 integral membrane proteins could be identified with significant Mowse scores. One integral membrane protein was identified after peptide sequence analysis using MALDI-MS and postsource decay analysis [5].

In previous studies we identified a large number of soluble proteins in extracytosolic compartments [2,26,28,29,60]. Recently, we carried out extensive multivariate amino acid sequence analyses of *Synechocystis* proteins routed for different compartments and showed that they have distinct and selective physicochemical properties in their essential signal peptide and mature N-terminals segments (Rajalahti *et al.*, unpublished manuscript). Including this study, we have now identified 40 integral plasma membrane proteins in *Synechocystis* [1,2], which in combination with known thylakoid membrane proteins from our own work [26] and that of others [24,51,61] provides a valuable platform for studies on membrane protein sorting. Multivariate analysis [62] of integral membrane proteins has been initiated in order to decrypt their address codes.

Experimental procedures

Cell culture and preparation of plasma membranes

The wild-type strain of *Synechocystis* sp. PCC 6803 was grown photoautotrophically at 30 °C under 60 $\mu E \cdot m^{-2} \cdot s^{-1}$ of white light in BG-11 medium [63]. Liquid culture was grown with vigorous air bubbling. The cells were harvested at $D_{750} = 2.0$. Plasma membranes from *Synechocystis* were purified by a combination of sucrose density centrifugation and aqueous two-phase partitioning [1,64].

Enrichment of integral membrane proteins and SDS/PAGE

The hydrophobic plasma membrane proteins were enriched by removing the peripheral proteins using urea. The pellet of plasma membranes (0.1 mg) was resuspended in 0.1 mL of 6.8 M urea/20 mM tricine–NaOH buffer (pH 8.0) and incubated at room temperature for 10 min followed by freezing on dry ice and thawing. The integral proteins from six membrane preparations were recovered as a pellet by centrifugation at 125 000 ***g*** for 15 min at 4 °C. Urea-washed membranes were pooled, suspended in solubilization buffer and loaded on a gradient SDS/PAGE (10–18%) according to Laemmli [65]. Reproducible Coomassie Brilliant Blue (R-250)-stained protein patterns were obtained for three gels (16 cm long) from different membrane preparations.

MALDI-TOF MS analysis and database search

Protein spots were cut out by OneTouch Plus Spot/Band picker using disposable tips (Gel Co., San Francisco, CA, USA). In-gel trypsin digestion and sample preparation were done manually as described previously [66]. The sample was then loaded onto a micropipette tip (C18 Zip Tip; Millipore, Bedford, MA), washed 10 times with 10 μL of 0.1% trifluoroacetic acid and followed by elution with 1 μL of 50% acetonitrile/0.1% trifluoroacetic acid. Analyses were conducted using α-cyano-4-hydroxycinnamic acid (10 $mg \cdot mL^{-1}$ in acetonitrile/0.1% trifluoroacetic acid 50:50 v/v) as the matrix, mixing equal volumes of the sample and the matrix and spotting 1 μL of the mixture on a standard stainless steel 96-sample MALDI target plate.

Peptides were analysed on an Applied Biosystems (Framingham, MA) Voyager-DE STR time-of-flight mass spectrometer equipped with a nitrogen laser (337 nm, 3 ns pulse width, 20 Hz repetition rate). All spectra were acquired in the reflectron mode with delayed extraction. External mass calibration was performed using low mass Peptide Mass Standards Kit (Applied Biosystems); the mass accuracy was typically < 20 p.p.m. Internal mass calibration was performed using trypsin 842.50 and 2211.10 Da autodigestion products and a matrix peak 1060.06 [67].

Proteins were identified based on the highest ranking results by searching through the National Center for Biotechnology Information (NCBI) database among all species using MASCOT (http://www.matrixscience.com/cgi/search_form.pl?FORMVER = 2 & SEARCH = PMF). The parameters applied for the Peptide Mass Fingerprint database search were: variable modification due to methionine oxidation, fixed modification due to carboamidomethylation of cysteines, one missed cleavage of trypsin and 70 p.p.m. mass accuracy. Measured peptides masses were excluded if their isotopic patterns were atypical or if their masses corresponded to trypsin autolysis products/matrix ions or adjacent identified proteins on the gel.

PSD fragment ion spectrum was obtained after isolation of the precursor ions using timed ion selection [5]. Fragment ions were refocused onto the final detector by stepping the voltage applied to the reflectron in the following ratios: 1.000 (precursor ion segment), 0.912, 0.750, 0.563, 0.422, 0.316, 0.237, 0.178, 0.133, 0.100 and 0.075 (fragment ion segments). The individual segments were stitched together using software developed by Applied Biosystems. The precursor ion segment was acquired at low laser power with < 256 laser pulses to avoid detector saturation. The laser power was increased for the remaining segments of the PSD acquisition. The PSD data were acquired at a digitization rate of 20 MHz; therefore, all fragment ions were measured as chemically averaged and not as monoisotopic masses. The resulting PSD mass spectrum was searched against the SWISS-PROT database. Search inputs included the measured precursor and fragment ion masses. Conservative error tolerances typically used were ± 100 p.p.m. for the precursor and ± 1 Da for the chemically averaged fragment ions.

Putative signal peptides and their cleavage sites were predicted using SIGNALP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) and TATP 1.0 (http://www.cbs.dtu.dk/services/TatP-1.0/). Transmembrane helices and lipoproteins were predicted using TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/) and LIPOP 1.0 (http://www.cbs.dtu.dk/services/LipoP/), respectively.

Acknowledgements

We thank Russell Schwartz (Carnegie Mellon University, Pittsburgh, USA) for the p*I* values, and Erik Granseth (Stockholm University, Sweden) for prediction of transmembrane helices and signal peptides of the genome. Support for this study was provided by the Carl Trygger and Mag. Bergvall Foundations.

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