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The cyanobacterium, *Synechococcus* sp. PCC7942, possesses two distinct genes encoding cation-transporting P-type ATPases

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P-type (or E1 E2-type) ATPases comprise a large family of prokaryotic and eukaryotic proteins capable of transporting a variety of cations, and function in a wide variety of cellular processes. The present study was carried out to search for genes encoding P-type ATPases in the phototrophic cyanobacterium, *Synechococcus* sp. PCC7942. We succeeded in cloning two genes each encoding P-type ATPases from this bacterium. It was found that *Synechococcus* at least, two distinct P-type ATPases; one belongs to the family of typical prokaryotic P-type ATPases and the other markedly resembles eukaryotic P-type ATPases. An insertion mutant lacking either of these two ATPase-genes was constructed. The results showed that the growth of these mutants is hypersensitive to osmotic stress upon addition of NaCl or sorbitol to the medium.

Cation transport; P-type ATPase; Cyanobacterium; Osmotic adaptation

1. INTRODUCTION

Cyanobacteria can be defined as microorganisms that harbor, within an otherwise typical prokaryotic cell, a photosynthetic apparatus similar in structure and function to that in the chloroplasts of phototrophic eukaryotes [1]. As simple bacteria, the cyanobacteria would seem to be the organisms of choice for the study of such fundamental processes as oxygen-evolving photosynthesis, inorganic carbon assimilation and nitrogen fixation ([2] and references therein). In this respect, changes in environmental growth conditions must greatly influence these internal physiological processes. Therefore, as widely recognized in free-living microorganisms, cyanobacterial cells must constantly monitor external conditions and adjust their structure and physiology accordingly. Osmotic adaptation in response to the external osmolarity is such a general adaptive response ([3] and references therein). With regard to the osmotic adaptation by a unicellular cyanobacterium (*Synechococcus* sp. PCC7942), we are particularly interested in potassium (K^+) ion transport systems, since the accumulation of K^+ ions is known to be the primary response to hyperosmotic stress (or changes in turgor pressure) in the best-characterized eubacteria such as *Escherichia coli* [3].

In *E. coli*, the accumulation of K^+ ions in response to hyperosmotic stress is mediated by a primary ATP-dependent pump (K^+-ATPase), which belongs to the family of P-type ATPases [4,5]. In cyanobacteria, however, the characteristics of K^+ transport systems are poorly understood in terms of the osmotic adaptation. Some physiological data are available for only a limited number of cyanobacterial strains [3,6,7]. In this respect, early studies on *Synechocystis* have suggested the occurrence of a primary active ATP-dependent K^+ uptake system that responds directly to changes in turgor pressure [7]. Thus, the present study was carried out to directly search for genes encoding P-type ATPases in *Synechococcus* sp. PCC7942. We succeeded in cloning two genes, and the results of analyses of their primary sequence verified that their products are indeed both P-type ATPases.

2. MATERIALS AND METHODS

2.1. *Bacterial strain and growth conditions*

Synechococcus sp. PCC7942 was kindly provided by Dr T. Omata (Nagoya University). This bacterium was photoautotrophically grown at 30°C in BG-11 medium (liquid or solid, containing 1.4% agar) [1]. For liquid cultivation, cultures were continuously aerated. When required, kanamycin was added at the concentration of 30 μg/ml.

2.2. *Enzymes and chemicals*

DNA-manipulating enzymes, including restriction endonucleases, and a BcaBEST sequencing kit were obtained from Takara Shuzo Co. or Toyobo Co [α-^{32}P]dCTP (3000 Ci/mmol) was purchased from Amersham International. Oligonucleotide primers, dGA(TC)AA(AG)AC(AGCT)GG(AGCT)AC(AGCT)CT and dG(CA)(AG)TC(AG)TT(AGCT)(AG)(TC)(AGCT)CC(AG)TC, were synthesized with an automated DNA synthesizer (the Center for Gene Research, Nagoya University).

2.3. *PCR-amplification*

The polymerase chain reaction was carried out with a Thermal Cycler (Perkin-Elmer Cetus) with Taq polymerase (Pharmacia). The conditions were those recommended by the suppliers

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Abbreviations: PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase (or 1000-bp); ORF, open reading frame.

2.4. *DNA techniques*

Recombinant DNA techniques including Southern transfer hybridization and phage plaque hybridization were all carried out according to conventional laboratory methods [8] For hybridization experiments, a non-radioactive DIG DNA labeling and detection kit (Boehringer Mannheim) was mainly used.

2.5. *DNA sequencing and analysis*

The sequencing of double-stranded DNAs on plasmid pUC119 was carried out by the dideoxy chain termination method [9] The generation of successively shortened DNA subclones for sequencing was performed using a kilo-sequence deletion kit (Takara Shuzo Co.). Analyses of nucleotide and amino acid sequences were carried out with a computer (NEC-PC9801) using the GENETYX program from Software Development Co.

2.6. *Construction of deletion mutants*

Synechococcus PCC7942 grown in BG-11 medium was harvested at the logarithmic growth phase, and incubated with appropriate linearized-plasmid DNAs (see Fig. 1). After the suspension had been stood under light for 10 h, the cells were spread on BG-11 agar plates containing kanamycin (30 μg/ml). After successive selection and single-colony-isolation, the colonies arising on this plate were isolated These procedures are essentially the same as those described previously [1]. To confirm that they were appropriate deletion mutants (*ΔpacS* or *ΔpacL*), Southern hybridization and PCR-amplification analyses was carried out with chromosomal DNAs prepared from these candidates A total chromosomal DNA from the putative *ΔpacS* strain was digested with *Hin*dIII or *Pvu*II, and then subjected to Southern hybridization with a *Bam*HI–*Xho*I fragment encompassing the *pacS*-coding sequence as a probe (see Fig. 1) Similarly, a total chromosomal DNA from the putative *ΔpacL* strain was digested with *Bam*HI/*Eco*RI or *Bam*HI/*Bgl*II, and then analyzed with a *Hpa*I–*Pst*I fragment encompassing the *pacL*-coding sequence as a probe (see Fig. 1).

3. RESULTS

All P-type ATPases exhibit a strikingly similar amino acid sequence, it being conserved among both prokaryotic and eukaryotic members of this family ([11] and references therein). Comparison of the amino acid sequences of this family enabled us to design a pair of degenerated oligonucleotide mixtures, which could be used for PCR-amplification. The two 17-mer oligonucleotides, we thus designed, correspond to the sequences, DKTGTL and DG(T/I/V)ND(A/S), respectively: the former (5′-primer) contains an autophosphorylation site characteristic of P-type ATPases and the latter (3′-primer) constitutes a part of the putative ATP binding region ([12] and references therein) (see Fig. 1). Using these primers, PCR-amplification was carried out for the *Synechococcus* total chromosomal DNA (Fig. 1, lane 1). Two DNA fragments (about 650-bp and 1000-bp in length) were reproducibly amplified under the conditions used. These two DNA fragments (tentatively named S and L, respectively) were isolated and cloned onto pUC119. Determination of the nucleotide sequences of these DNAs suggested that they could encode amino acid sequences each homologous to a portion of P-type ATPases. However, the amino acid sequences predicted for S and L were clearly different from each other. We then attempted to clone the entire genes by screening a *Synechococcus* sp. 7942 DNA library, constructed in λD ASH, with the respective DNAs (S and L) as probes. This yielded positive λ phages. The results of successive Southern hybridization analyses of these λ phage DNAs showed that 5.1-kb *Bgl*II–*Eco*RI and 4.7-kb *Eco*RI fragments hybridized with probe-S and probe-L, respectively (data not shown, see Fig. 1B and C). Each fragment was cloned onto pUC119 (plasmids pPAC-S and pPAC-L, respec-

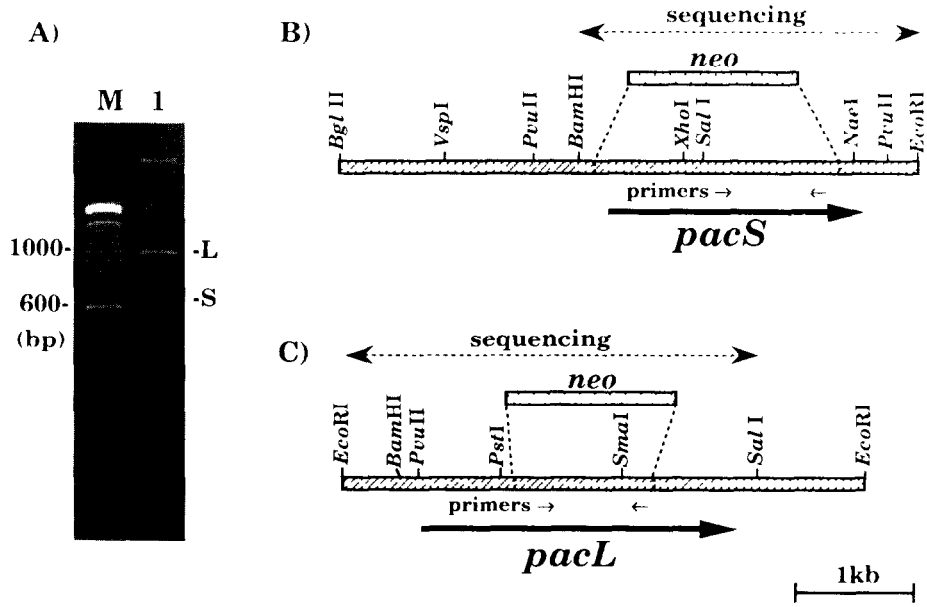

Fig. 1. Cloning of the genes encoding P-type ATPases from *Synechococcus* sp PCC7942. Panel A. A PCR-amplification profile on 2% agarose gel is shown in lane 1. Lane M represents a 100-bp DNA ladder-marker. Note that the amplified DNA fragments, denoted by S and L, were characterized in this study. Panel B. Schematic representation of the *Synechococcus* chromosomal region encompassing the *pacS* gene. Panel C. Schematic representation of the *Synechococcus* chromosomal region encompassing the *pacL* gene. Other details are given in the text

tively). Finally, the restriction maps of these cloned DNAs were determined, as shown in Fig. 1.

After construction of a set of appropriate subclones on the plasmids, both the *Bam*HI–*Eco*RI and *Eco*RI–*Sal*I regions were subjected to DNA sequencing, as indicated in Fig. 1 (the nucleotide sequence data will appear in the GenBank/EMBL/DDBJ Nucleotide Sequence Data Libraries under accession numbers D16437 and D16436, respectively). As schematically shown in Fig. 1, determination of the complete nucleotide sequence for pPAC-S revealed the presence of an ORF of 2241-bp, which could encode a protein consisting of 747 amino acids (panel B), whereas that determined for pPAC-L revealed the presence of an ORF of 2778-bp, which could encode a protein consisting of 926 amino

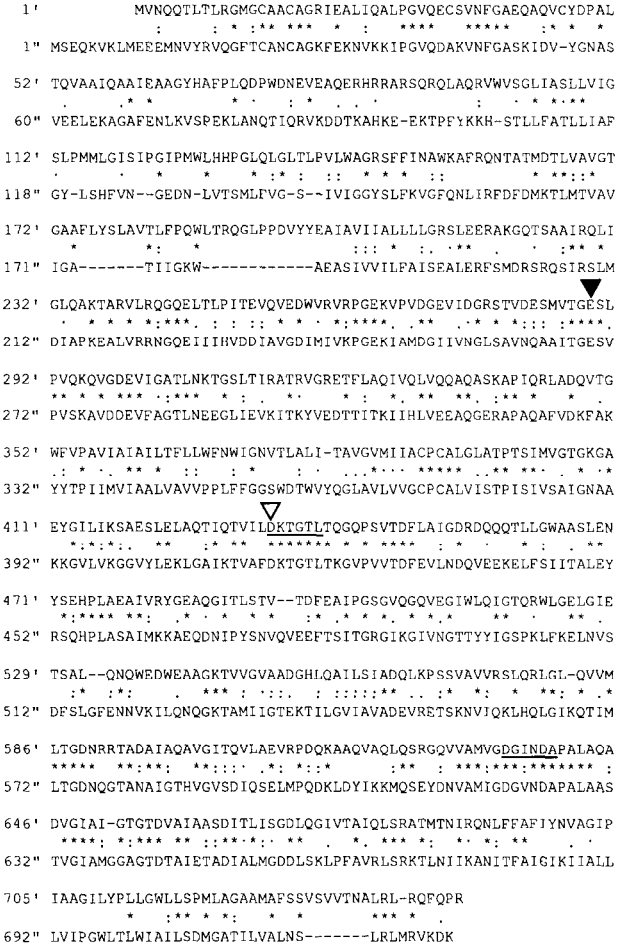

Fig. 2. The amino acid sequence of PacS in comparison with that of a typical prokaryotic P-type ATPase The amino acid sequence of PacS (upper line) was aligned with that of the Cd^{2+}-ATPase from *Sta. aureus* (lower line) [14] using the algorithm of Lipman and Pearson [24]. Note that the entire sequence is presented in both cases. An asterisk denotes an identical amino acid, whereas a colon denotes a conservative substitution The conserved amino acid stretches, which were used to design appropriate primers for PCR-amplification, are underlined. The invariant amino acids among all the P-type ATPases are indicated by triangles; the aspartate residue (D) is involved in autophosphorylation and the glutamate residue (E) in dephosphorylation.

Fig. 3. The amino acid sequence of PacL in comparison with that of a typical eukaryotic P-type ATPase. The amino acid sequence of PacL (upper line) was aligned with that of the PMR1 Ca^{2+}-ATPase from *Sac cerevisiae* (lower line) [15]. Other details are essentially the same as those given in the legend to Fig. 2

acids (panel C). The amino acid sequences deduced for these ORFs were compared with those of appropriate P-type ATPases (Figs. 2 and 3). This simple computer-aided inspection clearly indicated that both of these putative gene-products closely resemble P-type ATPases along their entire regions. It is known that members of the P-type ATPase family exclusively contain several invariant amino acid residues important for ATP binding, autophosphorylation and de-

phosphorylation [11]. Most of these residues were also identified in both the ORFs (see Figs. 2 and 3). Hence, the ORFs encoding 747 and 926 amino acids were tentatively named *pacS* and *pacL*, respectively (*P*- type *A*TPases of *c*yanobacteria).

The predicted amino acid sequences of both PacS and PacL suggested that they are integral membrane proteins which contain multiple hydrophobic regions capable of spanning the membrane many times, as has been pointed for P-type ATPases. Prokaryotic members of the P-type ATPase family, for which predicted amino acid sequences have been reported, are the KdpB subunit of the *E. coli* K^+-ATPase [4], an ATPase from *Streptococcus faecalis* [13], a plasmid-encoded Cd^{2+} ATPase (*Staphyrococcus aureus*) [14], an ATPase from *Rhizobium meliloti* [12], and a Mg^{2+}-ATPase from *Salmonella typhimurium* [11]. As shown in Fig. 3, optimal alignment of the predicted amino acid sequence of PacS with that of the *Sta. aureus* Cd^{2+}-ATPase indicated 32% amino acid identity plus 25% conservative substitutions, the overall similarity being 57% along the entire lengths. The similarity to the ATPases from either *Str. faecalis*, *R. melilotti*, or *E. coli* is also significantly high (data not shown). The prokaryotic P-type ATPases described previously are approximately 750 amino acids in length, which is in good agreement with the lenght of PacS (747 amino acids). From these results, PacS was concluded to be a typical prokaryotic P-type ATPase, although its cation specificity is not known.

PacL comprises 926 amino acids in length, which is much larger than other prokaryotic P-type ATPases. PacL is about the same size as eukaryotic P-type ATPases, which are generally 900 to 1200 amino acids in length. In fact, PacL is most similar along the entire lenght to the PMR1 Ca^{2+}-ATPase from *Saccharomyces cerevisiae* [15]. As shown in Fig. 4, optimal alignment of the predicted amino acid sequence of PacL with that of the PMR1 Ca^{2+}-ATPase indicated 35% amino acid identity plus 23% conservative substitutions, the overall similarity being 58%. Such a high degree of similarity was also seen with Ca^{2+}-ATPases of mammalian sarcoplasmic reticulum [16] and Na^+/K^+-ATPases from mammals [17] (data not shown). PacL is somewhat less similar to eukaryotic H^+-ATPases, including from plants [18,19]. However, the similarity is still greater than the similarity to prokaryotic P-type ATPases, i.e. the similarity of PacL to prokaryotic ATPases was markedly less. In this respect, it has been reported that the *Sal. typhimurium* Mg^{2+}-ATPase is more similar to eukaryotic ATPases than it is similar to prokaryotic ATPases [11]. However, our computer-aided alignment analyses indicated that PacL is significantly more similar to eukaryotic ATPases (data not shown). In any case, it was revealed that *Synechococcus* sp. PCC7942 has a P-type ATPase that is very similar to eukaryotic ATPases, although its cation specificity is not known.

To obtain clues with regard to the physiological function of the *pacS* and *pacL* genes, deletion mutants of *Synechococcus* sp. PCC7942 were constructed in which a large portion of each coding-sequence was replaced by the *neo* (kanamycin-resistance) gene on the chromosome, as shown in Fig. 1. Most of the coding sequence for *pacS* or *pacL* was deleted in these mutations (*Δ*(Met-1 to Thr-686) for PacS, *Δ*(Leu-271 to Val-685) for PacL). When these mutants were grown in the conven-

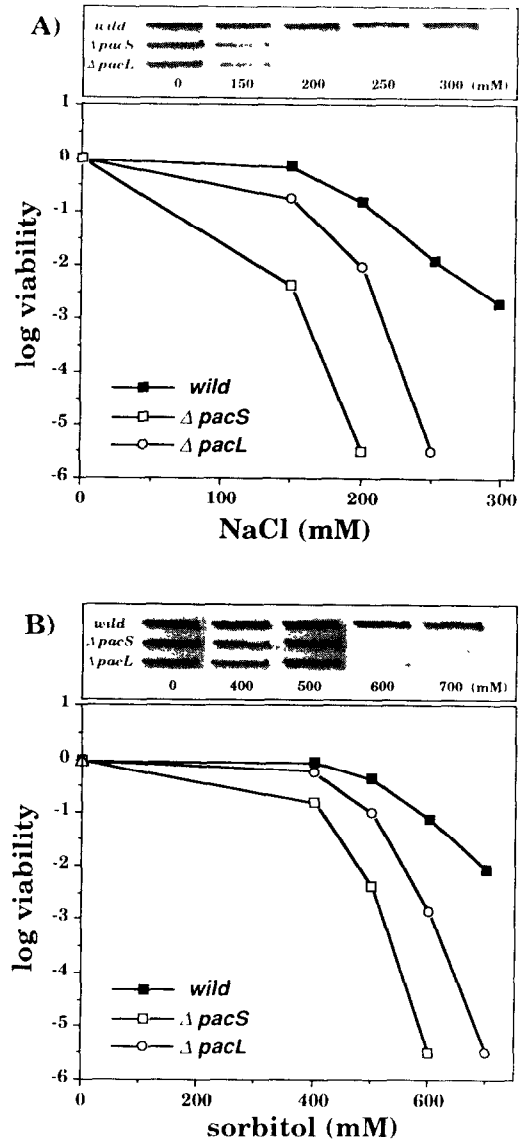

Fig. 4. Growth ability of *Synechococcus* sp. PCC7942 mutants lacking either the *pacS* or *pacL* gene. The *ΔpacS* and *ΔpacL* strains, as well as their parental wild-type strain, were streaked on solid BG-11 medium containing different concentrations of NaCl (Panel A) or sorbitol (Panel B), as indicated. After incubation for 135 h at 30°C, pictures of the plates were taken. Similarly, these mutants grown on BG-11 medium were serially diluted with fresh BG-11 medium, and then spread on plates containing different concentrations of NaCl (Panel A) or sorbitol (Panel B). After incubation for 135 h at 30°C, the number of colonies was determined and expressed relative to the colony number on control plates supplemented with neither NaCl nor sorbitol.

tional BG-11 medium, we were not able to find any noticeable phenotype for either deletion mutant (*ΔpacS* or *ΔpacL*) (see Fig. 4). These mutants were also grown in BG-11-based media each supplemented with varied concentrations of cations/metals, namely, Mg^{2+} (up to 150 mM), Ca^{2+} (up to 100 mM), Co^{2+} (up to 1.6 μM), Zn^{2+} (up to 10 μM), Hg^{2+} (up to 2 μM), Cd^{2+} (up to 2 μM) and Cu^{2+} (up to 4 μM). They were also grown in BG-11-based media each depleted cations, namely K^+ (5 μM), Mg^{2+}(5 μM) and Ca^{2+} (0.5 μM). When we compared their phenotypes for growth in these media with those exhibited by the wild type, we were unable to find any noticeable phenotypic alterations (data not shown). However, it is worth mentioning that the *ΔpacS* mutant appeared to be somewhat hypersensitive to Cu^{2+} metal (data not shown).

Then, these deletion mutants were examined for their ability to grow in hyperosmotic medium, it was found that the *ΔpacS* strain grew very poorly on solid BG-11 medium containing relatively high concentrations of NaCl (200–300 mM) or sorbitol (600–700 mM) (Fig. 4A and B, respectively). To quantitatively examine this further, the colony-forming ability on the hyperosmotic solid medium was also examined (Fig. 4A for NaCl; Fig. 4B for sorbitol). The results indicated that the strain lacking the *pacS* gene appears to be hypersensitive to hyperosmotic growth conditions. It is worth mentioning that when the *pacS* gene on an *E. coli–Synechococcus* shuttle vector was introduced into the *pacS* deletion mutant, the resultant transformant grew normally even on the hyperosmotic medium (data not shown). The *ΔpacL* strain also exhibited essentially the same phenotype, but its hypersensitivity to the hyperosmotic medium was less evident, as shown in Fig. 4. Thus, it is probable that these P-type ATPase genes, particularly *pacS*, may be involved, either directly or indirectly, in the osmotic adaptation of *Synechococcus* cells.

4. DISCUSSION

In this study, we succeeded in cloning two distinct genes each encoding P-type ATPases in *Synechococcus*. One of the ATPases (PacS) appears to be a typical prokaryotic P-type ATPase. We found that the strain lacking this *pacS* gene is hypersensitive to hyperosmotic growth conditions. As mentioned above (see section 1), it has been reported for some cyanobacteria that a rapid increases in the intracellular K^+ concentration occurs as a direct consequence of hyperosmotic treatment, in which an energy (ATP)-dependent K^+ transport system appears to be involved [7]. PacS may play a role in the osmotic adaptation by mediating osmotic inducible K^+ influx, as has been well established in *E. coli* [3]. However, it should be also noted that some prokaryotic P-type ATPases have been suggested to be involved in metal-efflux, e.g. a *Sta. aureus* ATPase for Cd^{2+} [12] and a *Str. faecalis* ATPase for Cu^{2+} [20]. In this respect, it is worth mentioning that the *ΔpacS* mutant appears to be somewhat hypersensitive to Cu^{2+}. In any case, the cation specificity of PacS should be biochemically determined in order to gain a clue with regard to the physiological function of PacS in *Synechococcus*. These must await further experiments.

The other P-type ATPase (PacL) is also intriguing, and the reason is two-fold. First, as far as the primary amino acid sequence is concerned, PacL clearly belongs to the eukaryotic ATPase family. In particular, PacL is most similar to some eukaryotic Ca^{2+}-ATPases [15,16]. Second, it is thus tempting to suppose that PacL may be a Ca^{2+}-ATPase, although its cation specificity has not yet been determined. In eukaryotes, including plants, the regulation of the intracellular Ca^{2+} level is widely recognized as a central element of various regulatory processes, in which P-type Ca^{2+}-ATPases play important roles. In this respect, prokaryotic Ca^{2+}-ATPases are thought to be extremely uncommon (bacteria appear to favor Ca^{2+} transport via secondary transport systems). However, there are several biochemical indications of the occurrence of primary Ca^{2+}-ATPases, which have been reported for some bacterial species [20–22]. Therefore, Ca^{2+}-ATPases may play an important physiological role(s) also in certain prokaryotic cells, and PacL may be such a Ca^{2+}-ATPase. In any case, it would be of interest to determine why *Synechococcus* possesses a eukaryotic-type ATPase. Finally, this fact is also intriguing from an evolutional point of view.

Among prokaryotes and eukaryotes, P-type ATPases are implicated in a wide variety of cellular processes. In prokaryotes, however, only a limited number of members of this family have been characterized at the molecular level [4,11–14]. In conclusion, this is the first report of the molecular cloning of two distinct genes encoding P-type ATPases in a phototrophic prokaryote. We are currently examining PacS with special reference to osmotic adaptation, as discussed above. PacL is currently being examined with special reference to the photosynthetic ability of *Synechococcus*, since this eukaryotic-type ATPase may be relevant to the eukaryotic ability as to photysynthesis exerted by this particular bacterium.

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