**Abstract**

High and changing salt concentrations represent major abiotic factors limiting the growth of microorganisms. During their long evolution, cyanobacteria have adapted to aquatic habitats with various salt concentrations. High salt concentrations in the medium challenge the cell with reduced water availability and high contents of inorganic ions. The basic mechanism of salt acclimation involves the active extrusion of toxic inorganic ions and the accumulation of compatible solutes, including sucrose, trehalose, glucosylglycerol, and glycine betaine. The kinetics of these physiological processes has been exceptionally well studied in the model *Synechocystis* 6803, leading to the definition of five subsequent phases in reaching a new salt acclimation steady state. Recent ‘omics’ technologies using the advanced model *Synechocystis* 6803 have revealed a comprehensive picture of the dynamic process of salt acclimation involving the differential expression of hundreds of genes. However, the mechanisms involved in sensing specific salt stress signals are not well resolved. In the future, analysis of cyanobacterial salt acclimation will be directed toward defining the functions of the many unknown proteins upregulated in salt-stressed cells, identifying specific salt-sensing mechanisms, using salt-resistant strains of cyanobacteria for the production of bioenergy, and applying cyanobacterial stress genes to improve the salt tolerance of sensitive organisms.

**Introduction**

Cyanobacteria represent an ancient monophyletic phylum of the domain Bacteria. In general, it is assumed that these organisms developed oxygenic photosynthesis 2.5–3.5 billion years ago and later this ability was transferred to eukaryotes via the endosymbiosis of an ancient cyanobacterium, leading to the emergence of eukaryotic algae and plants (Mereschkowsky, 1905; Margulis, 1970). Recent genome analyses seem to indicate that the primary endosymbiont was most closely related to present-day filamentous strains (Deusch et al., 2008). The photoautotrophic lifestyle made cyanobacteria dependent only on light, water, and a few inorganic nutrients. As a consequence, cyanobacteria became dominant organisms with major impacts on geochemical processes of our planet. During their massive development, cyanobacteria generated the oxygen-containing atmosphere, which gave rise to the evolution of most oxygen-dependent organisms found today. The long time elapsed since their origin has allowed cyanobacteria to adapt to almost all habitats with at least minimal light. This broad range of habitats mirrors the impressive ability of cyanobacteria to acclimate to changes of many different environmental factors (Tandeau de Marsac & Houmard, 1993).

Cyanobacteria are also still very important players in biogeochemical cycles; it was estimated that they account for about 30% of global primary production, especially in the central oceans, which make them an important sink for CO₂. Additionally, because of their N₂-fixing ability, many cyanobacterial strains are regarded as the major sources of combined nitrogen in the marine system (for references see Scanlan et al., 2009). They represent a unique bacterial phylum that shows many differences from classical Gram-negative bacteria such as *Escherichia coli*, including cell wall structure (Gupta & Griffiths, 2002). Like Gram-negative bacteria, cyanobacteria are surrounded by two membrane systems. The cell wall is enclosed by an outer membrane that surrounds the periplasmic space, whereas the cytoplasmic membrane surrounds the cytoplasm. Inside the cytoplasm, cyanobacteria contain a third membrane system. Thylakoid membranes, which originate from the cytoplasmic membrane, contain the photosynthetic complexes. In addition,
the respiratory electron transport chain is also mostly situated at the thylakoid membranes.

Salinity, the total concentration of inorganic ions, is a very important environmental factor, especially for aquatic organisms, such as most cyanobacteria. Cyanobacterial salt acclimation has rarely been reviewed. Our last comprehensive overview was in 1996 (Joset et al., 1996). The biosynthesis of compatible solutes among cyanobacteria was briefly reviewed in the last decade (Erdmann et al., 1999; Hagemann et al., 1999b; Erdmann & Hagemann, 2001; Karandasheva & Elanskaya, 2005). Recently, reviews were published on ecological aspects of salt-exposed cyanobacteria (Oren, 2000, 2007) as well as the effects of salt on the photosynthetic apparatus (Allakhverdiev & Murata, 2008). The principles of salt acclimation and possible sensing mechanisms were reviewed by Wood (1999). The basics of the synthesis and uptake of compatible solutes among heterotrophic bacteria have been described by Bremer & Krämer (2000). The present review reports comprehensively on the physiological mechanisms and underlying molecular events that allow cyanobacteria to live in environments with different and changing salt concentrations.

Changing salt concentrations – the problem

Increased salt concentrations of the surrounding medium challenge organisms in two ways: first, the water potential is lower, and secondly, high concentrations of some ions can have toxic effects on cellular metabolism. Water is taken up in all living cells by osmosis, which is driven by the water potential gradient. A cell can take up water when it has a lower water potential than the surrounding medium. However, ion concentrations of about 100 mM NaCl (equivalent to an osmotic concentration of about 200 mOsmol L\(^{-1}\)) lower the water potential in the medium to an extent that makes it difficult for most cells to obtain or even retain water without starting certain acclimation processes. To keep a constant water content, which is necessary to maintain turgor as the driving force for growth, the cytoplasmic concentration of osmotically active compounds must be higher than that of the surrounding medium (e.g. Ladas & Papageorgiou, 2000). One possibility is the uptake of ions from the medium, which is described by the ‘salt-in’ strategy. The salt-in strategy can be found in some highly specialized microorganisms able to propagate in nearly saturated brines, i.e. by some halophilic Archaea and halophilic anaerobic as well as a few aerobic Bacteria. These prokaryotes tolerate 2–3 M salt concentrations (mostly KCl) inside the cytoplasm. Their metabolism has adapted to the presence of such high salt concentrations over the course of evolution, resulting in growth limitation in the absence of such high concentrations (Müller & Oren, 2003). Only a few organisms are known to perform the salt-in strategy, and they are restricted to rare ecological niches. The majority of cells/organisms use the ‘salt-out’ strategy. Using this strategy, cells keep a rather constant but low internal concentration of inorganic ions. Lowering the water potential in the presence of high external salinity is mainly achieved by the accumulation of compatible solutes (Bremer & Krämer, 2000). The concept of compatible solutes was introduced about 35 years ago. As the name implies, they are low molecular mass, highly soluble organic compounds which can be accumulated in high concentrations in the cytoplasm without interfering with metabolism (i.e. they are ‘compatible’ with the cell metabolism) (Brown, 1976). These compounds are synthesized de novo or taken up from the environment (Bremer & Krämer, 2000). At high internal ion concentrations, especially those of Na\(^+\) and Cl\(^-\), cells using the salt-out strategy are negatively affected because metabolic activities are inhibited by higher salt concentrations. The inhibition can be due to different primary effects, but often the hydration shell or surface charge is affected by high concentrations of noncompatible inorganic ions. To keep ion concentrations constant, ions are actively extruded at hypersaline conditions but must be imported at hyposaline conditions (Wood, 1999). Salt stress is often referred to as osmotic stress. However, in the strictest sense, osmotic stress is caused by the addition of organic, nonpermeable solutes to the medium, which also affect the water potential but do not produce ionic stress. Drought or desiccation stress is also known to extract water from cells. When water is lost from a cell, the internal ion composition is affected. Therefore, there is some overlap in the acclimation of cyanobacteria to salt as well as to osmotic and drought stresses; however, in the present review we will focus mainly on work on salt stress acclimation and only sometimes related work on true osmotic or desiccation stress.

Salt acclimation

Inorganic ion homeostasis

As mentioned above, cyanobacteria occur in environments with different salinities. Usually, high salt means an increase in the external concentration of NaCl, the quantitatively dominant salt in seawater, which is therefore most used in laboratory experiments. There are many reports showing that the different ion composition of alkaline lakes or hypersaline environments, where other ions predominate, may pose additional challenges to their inhabitants; therefore, only highly specialized organisms, including some specialized cyanobacteria, can live in such extreme environments (Oren, 1999; Nübel et al., 2000). Here, processes will be described that are involved in acclimation to elevated Na\(^+\) and Cl\(^-\) concentrations and the role of K\(^+\), which is an important monovalent cation inside the cell, where it is...
preferentially accumulated and exchanged with the toxic Na\(^+\). The often observed toxicity of the alkaline ion Na\(^+\) and the compatibility of the alkaline ion K\(^+\) are not well understood (Maathuis & Amtmann, 1999). Both ions show similar physicochemical structures, as the smaller ion Na\(^+\) together with its rather large hydration shell mimics the size of K\(^+\). Therefore, uptake systems for K\(^+\) have difficulties discriminating between these ions, and high Na\(^+\) contents in the medium may result in K\(^+\) deficiency. Inside the cell, Na\(^+\) competes for K\(^+\)-binding sites [e.g. K\(^+\) is often found in proteins stabilizing and defining three-dimensional (3D) structures], resulting in the inhibition of K\(^+\)-dependent metabolic processes. Therefore, all organisms tend to ensure a defined K\(^+\)/Na\(^+\) ratio in the cytoplasm (Maathuis & Amtmann, 1999).

**Na\(^+\) content and transport**

Na\(^+\) is the main inorganic cation in oceans and in most other saline environments. Along the electrochemical gradient, Na\(^+\) enters cells under hypersaline conditions. As high cytoplasmic concentrations of Na\(^+\) are toxic, Na\(^+\) concentrations are much lower inside salt-stressed cyanobacterial cells than in the surrounding medium (Table 1). Unfortunately, it is rather difficult to obtain reliable data on the intracellular Na\(^+\) concentrations of salt-treated cyanobacterial cells. The first and main problem in collecting such data is the elimination of Na\(^+\) contamination from the salt-rich external medium. The necessary washing procedures may extract not only contaminating or loosely bound Na\(^+\) from the cell wall but some of the internal Na\(^+\) as well. The second problem is distinguishing Na\(^+\) in the periplasm or cytoplasm from bound or freely dissolved sodium ions. The third problem is accurately estimating the osmotic volume of the cyanobacterial cell, which is usually not known. Therefore, ion concentrations are mostly given on a total cell volume basis, including the cell wall, membranes, periplasm, and all macromolecules inside the cell. Accurate estimations of the osmotic volume can be obtained using radiotracers, which have access only to specific cell compartments, or using the ESR technique, following the distribution of different permeable spin-labels (Blumwald et al., 1983a, b; Ritchie, 1991; Nitschmann & Packer, 1992). Unfortunately, there are only a few cases in which authors have reported the cytoplasmic volume in relation to the total cell volume measured, for example by a Coulter counter. For *Anabaena* strains, it has been mentioned that the intracellular volume is about 40% of the packed total cell volume (Apte & Thomas, 1986). For *E. coli*, it was found that about 40% of the cytoplasmic volume is occupied by cellular constituents (ribosomes, etc.) and the free, accessible water space is restricted to a rather small part of the cell volume (Record et al., 1998a).

Of the published data on the Na\(^+\) content of salt-treated cyanobacterial cells, the most reliable values were obtained using tracer experiments with radio-labeled Na\(^+\), as this

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Extracellular NaCl concentration (mM)</th>
<th>Intracellular Na(^+) concentration (mM)</th>
<th>Intracellular K(^+) concentration (mM)</th>
<th>Methods</th>
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<tr>
<td><em>Synechococcus</em> PCC 6311</td>
<td>8.8</td>
<td>8</td>
<td>170</td>
<td>AAS</td>
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<td>300</td>
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<td>NMR</td>
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<td>Silicon oil centrifugation and FP</td>
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ND, not determined; AAS, atomic absorption spectroscopy; FP, flame photometry.
strategy allows the distinction of internal and external Na$^+$ (Reed et al., 1984a, 1985a, b; Ritchie, 1992a). These data show that cells grown in basal medium (e.g. BG11) contain about 5–10 mM Na$^+$, whereas salt-acclimated cells have sodium ion contents of 10–20% of the external Na$^+$ concentration (Table 1). These values are supported using in vivo nuclear magnetic resonance (NMR), another non-invasive technology that allows ion quantification in the cytoplasmic space without washing (Nitschmann & Packer, 1992). Using silicone oil centrifugation and flame photometry, we found a stronger increase of Na$^+$ in salt-acclimated cells of Synechocystis 6803, which can be attributed to the difficulty in completely removing external sodium ions (Hagemann et al., 1994). Nevertheless, all datasets show clearly that cyanobacteria [even when grown under hypersaline conditions as shown by Reed et al. (1984a)] contain significantly lower internal sodium concentrations compared with the external medium, suggesting active extrusion of this toxic ion from the cytoplasm.

The existence of quick ion transport processes was shown by kinetic studies, in which changes in Na$^+$ content were monitored following a hypersaline shock. In the first minutes after salt addition, an immediate rise in the internal Na$^+$ content was measured in cells of Synechococcus 6301 (Nitschmann & Packer, 1992) and Synechocystis 6714 (Reed et al., 1985b). However, within the next 20–60 min, the Na$^+$ content rapidly decreased to levels similar to those in long-term salt-acclimated cells. Obviously, the steep electrochemical gradient drives Na$^+$ immediately after shock (probably using nonspecific channels); however, at the same time, Na$^+$ export mechanisms become activated and extrude the toxic ions from the metabolically active part of the cell. Moreover, these studies indicated that a certain threshold of salt difference must be exceeded before such an ion influx can occur. This threshold is roughly 300 mM NaCl (Reed et al., 1985a). The very quick response in Na$^+$ export points to a direct activation of preformed export systems rather than new expression of transporters. This view is supported by the lack of gene inductions for any potential ion export system in salt-shocked cells of Synechocystis 6803 (Kanesaki et al., 2002; Marin et al., 2004). Finally, it is not known whether there are different Na$^+$ concentrations in the cytoplasmic and lumenal spaces in a cyanobacterial cell.

The low Na$^+$ concentrations observed inside salt-treated cells clearly indicate that active sodium ion export mechanisms exist in cyanobacterial cells. The bioenergetics of Na$^+$ extrusion was intensively characterized for the slightly salt-tolerant strain Synechococcus 7942. Ritchie (1992a) concluded that Na$^+$ export is mainly driven by the proton gradient; however, under alkaline conditions, a primary active Na$^+$ export mechanism was predicted. Na$^+$/H$^+$ antiporters are still the best candidates for sodium ion export, as was found earlier for E. coli (Padan & Schuldiner, 1993). The exchange of Na$^+$ with protons was observed early on in salt-treated cells of Synechococcus 6301 (Blumwald et al., 1984). However, these kinetic studies did not allow the elucidation of whether these two ions are exchanged by one protein or through the cooperation of two different proteins specific for each of the two ions. In the complete genome sequence of Synechocystis 6803, six different genes were annotated as Na$^+$/H$^+$ antiporters (Kaneko et al., 1996). Multiple genes for Na$^+$/H$^+$ antiporters are also present in other completely known cyanobacterial genomes (see Billini et al., 2008). The occurrence of rather large gene families for Na$^+$/H$^+$ antiporters might indicate that these proteins fulfill many important functions in cyanobacterial cells (Fig. 1).

To analyze the function of the Na$^+$/H$^+$ antiporter gene family, two strategies were mainly used: inactivation of single or multiple genes and overexpression of cyanobacterial genes in defined E. coli mutants. The latter strategy revealed that at least three antiporters from Synechocystis 6803 – NhaS1, NhaS3, and NhaS4 – are true antiporters, as they exhibited Na$^+$/H$^+$ antiporter activity in E. coli (Inaba et al., 2001). Among them, NhaS3 showed the highest transport activity and was able to restore tolerance to Na$^+$ as well as Li$^+$ in a salt-sensitive E. coli mutant defective in Na$^+$/H$^+$ antiporter activity. In an independent study, the NhaS1 antiporter, which shows similarities to the SOS1 Na$^+$/H$^+$ antiporter from Arabidopsis thaliana, was also expressed in E. coli, leading again to the restoration of a Na$^+-$ as well as Li$^+$-resistant phenotype (Hamada et al., 2001). The inactivation of single and multiple antiporter genes in Synechocystis 6803 revealed that most of the genes could be deleted without drastically reducing the salt or pH tolerance of these mutants (Elanskaya et al., 2002; Wang et al., 2002). These findings would seem to indicate that these antiporters are redundant. However, the gene for NhaS3 was found to be essential for cell viability and could not be completely deleted from mutant cells; only a reduction in its gene dosage was possible. As mentioned before, this protein seems to be the most active Na$^+$/H$^+$ antiporter in Synechocystis 6803 (Inaba et al., 2001), and the gene encoding it (sll0689) was the most highly expressed of the Na$^+$/H$^+$ antiporter genes (Elanskaya et al., 2002; Marin et al., 2004). This particular antiporter probably performs the majority of Na$^+$ export under saline conditions. This view is supported by the finding that the nonsegregated NhaS3 mutant with strongly reduced Na$^+$/H$^+$ antiporter activity also showed a salt-sensitive phenotype (Wang et al., 2002; Tsunekawa et al., 2009). Similar results were recently shown for the slightly salt-tolerant strain Synechococcus 7942. The closest homolog to NhaS3 was also found to be essential for cell viability and is most probably crucial for the salt tolerance of this strain (Billini et al., 2008). However, the recent study on NhaS3 from Synechocystis 6803 revealed that this antiporter is mainly localized to the thylakoid and
not to the cytoplasmic membrane (Tsukewaka et al., 2009), which makes its primary role in Na⁺ net export from salt-stressed cells questionable. Therefore, the authors suggested that its main role is in ion homeostasis between the cytoplasmic and lumenal spaces, which is dramatically changing, for example during light/dark cycles. This hypothesis is in line with findings on the regulation of the NhaS3 protein level by different CO₂ and light regimes, while rather low expression changes were observed in salt- or osmotically stressed cells (Tsukewaka et al., 2002; Wang et al., 2002; Billini et al., 2008).

Na⁺/H⁺ antiporters were also characterized in the hypersaline strain Aphanothece halophytica. As previously found for freshwater and moderately halotolerant strains, this cyanobacterium harbors multiple Na⁺/H⁺ antiporter genes (Waditee et al., 2001; Wutipraditkul et al., 2005). Initially, Waditee et al. (2001) cloned and characterized a Na⁺/H⁺ antiporter from A. halophytica, called ApNhaP, which is related to NhaS1 from Synechocystis 6803. However, the Na⁺/H⁺ antiporter protein from the halophilic strain exhibited an enlarged C-terminal tail, which was shown to alter its ion specificity. Beside Na⁺, this protein was able to exchange Ca²⁺ with H⁺ but not Li⁺ in defined E. coli mutants. The Na⁺/H⁺ antiporter Ap-NapA1-1, which shows the highest sequence similarities to NhaS3 from Synechocystis 6803, was able to restore the Na⁺ resistance as well as the Li⁺ resistance of the E. coli nha mutant. Interestingly, this Na⁺/H⁺ antiporter was most active at alkaline pH, characteristic for hypersaline environments. Moreover, a second Na⁺/H⁺ antiporter of the Nap family, called Ap-NapA1-2, was found in A. halophytica. In addition to Na⁺/H⁺ exchange activity, the Ap-NapA1-2 also showed a remarkable ability to take up K⁺, indicating that it might function as a Na⁺/K⁺ antiporter (Wutipraditkul et al., 2005). The expression of two genes encoding the Na⁺/H⁺ antiporters ApNhaP and Ap-NapA1-1 from A. halophytica in the freshwater strain Synechococcus 7942 increased its salt resistance significantly, which was taken as a further indication that these Na⁺/H⁺ antiporters promote high salt tolerance in A. halophytica (Waditee et al., 2002; Wutipraditkul et al., 2005). Moreover, this result suggests that the salt resistance of the freshwater strain Synechococcus 7942 is mainly limited by its ability to keep a low internal Na⁺ content.

In addition to exporting Na⁺ at enhanced salinities, a special group of Na⁺/H⁺ antiporters also functions in Na⁺ uptake when cyanobacterial cells are grown at low external Na⁺ or in media showing a rather high K⁺/Na⁺ ratio. Mutants of Synechocystis 6803 defective in the NhaS2 (Sll0273) did not show any difference in their tolerance to high salt concentrations but were unable to propagate at Na⁺ contents < 1 mM (Mikkat et al., 2000b; Wang et al., 2002). However, more detailed analyses showed that this growth defect was not solely dependent on the total Na⁺ supplement but rather on the ratio of Na⁺ to K⁺. A K⁺/Na⁺ ratio higher than 2 was found to be toxic for the NhaS2 mutant, i.e. the total requirement for Na⁺ increased at higher K⁺ concentrations and vice versa (Mikkat et al., 2000b). A similar phenotype was observed for a Synechococcus 7942 mutant defective...
in the NhaS2 homolog (Billini et al., 2008). These particular Na\(^{+}\)/H\(^{+}\) antiporters are probably necessary in low-salt medium for ion homeostasis.

Another possible transporter involved in Na\(^{+}\) extrusion is the Mrp system (monovalent cation/proton antiporter system). In contrast to Na\(^{+}\)/H\(^{+}\) antiporters, which are functional transporters made by a single protein, Mrp systems are proposed to function as hetero-oligomers. Correspondingly, they are encoded by operons consisting of six or seven genes, which code for different hydrophobic proteins cooperating in the export of sodium or lithium ions in many bacteria, including alkaliphilic species (Swartz et al., 2005). Defined mutants in any of these genes in, for example Bacillus strains, showed a decreased ability to grow at increased NaCl concentrations and/or under alkaline conditions. However, the exact function of the Mrp system is not well understood. Some of the subunits resemble membrane proteins of ion-translocating electron transport complexes, such as those found in the respiratory chain (Swartz et al., 2005). A related system was discovered and functionally characterized in the filamentous cyanobacterium Nostoc (Anabaena) 7120 by a transposon-mutagenesis screen for the isolation of alkali-sensitive mutants. Defined mutations in this gene or in other genes of the putative Nostoc 7120 mrp operon resulted again in a salt- and alkali-sensitive phenotype (Blanco-Rivero et al., 2005). Very recently, the mrp gene cluster was also identified in the halophilic strain A. halophytica (Fukaya et al., 2009). These authors showed that it functions as a Na\(^{+}\)(Li\(^{+}\))/H\(^{+}\) antiporter by expressing it in a corresponding E. coli mutant. However, its affinity for Na\(^{+}\) was lower than that reported for a classical Na\(^{+}\)/H\(^{+}\) antiporter from this strain. As A. halophytica is not amenable to directed mutation, the corresponding gene cluster was inactivated in the slightly halotolerant cyanobacterial strain Synechococcus 7942, resulting in a decreased salt tolerance, especially under alkaline conditions (Fukaya et al., 2009). Interestingly, genes for mrp operons are present in many genomes of cyanobacteria from different environments. Genome searches (at Cyanobase: http://blast.kazusa.or.jp/blast_search/cyanobase/genes) show that probably all of the rather large cyanobacterial genomes harbor an mrp operon. However, among the marine Synechococcus and Prochlorococcus strains, which are characterized by smaller, reduced genomes (Kettler et al., 2007), the mrp operon seems to be restricted to a few Synechococcus strains, and it is probably absent from all Prochlorococcus strains.

Generally, the energetics of Na\(^{+}\) export is not well resolved. It is believed that Na\(^{+}\)/H\(^{+}\) antiporters use the proton motive force or the membrane potential at the cytoplasmic membrane for the uphill export of Na\(^{+}\) in saline medium. Many studies have shown that H\(^{+}\)-pumping activity is directly stimulated in intact salt-treated cyanobacterial cells and even in isolated cytoplasmic membrane vesicles. These results were reported for cyanobacteria from different salt tolerance groups and seem to represent a common scheme in the salt acclimation process. The direct salt-stimulated extrusion of protons was attributed to an increased respiratory activity or stimulated activities of P-type H\(^{+}\)-ATPases (Nitschmann & Peschek, 1985; Molitor et al., 1986; Gabbage-Azaria et al., 1992, 1994; Jeanjean et al., 1993; Wiangnon et al., 2007). Accordingly, mutations of the terminal oxidase of the respiratory chain and of P-type ATPases resulted in reduced salt tolerance (Kanamaru et al., 1993; Ryu et al., 2003). The direct involvement of the respiratory electron transport in the establishment of the proton gradient was discussed, as the respiratory chain is not always located at the cytoplasmic membrane but, in some strains, at the thylakoid membrane (Peschek et al., 1994).

Another possibility for how respiration could be involved in salt acclimation is related to its function as a valve for the photosynthetic electron transport chain. In salt-treated cells, the photosystem I/II (PSI/PSII) ratio increased (Schubert & Hagemann, 1990) during the activation of cyclic electron transport activity around PSI (Fig. 1), which mainly occurs via the NDH1 complex and employs flavodoxin as well as a thylakoid-bound form of ferredoxin-NADP(H) oxidoreductase (Jeanjean et al., 1993, 1998; Hibino et al., 1996; Tanaka et al., 1997; Hagemann et al., 1999a; van Thor et al., 2000; Ooyabu et al., 2008). An increased cyclic electron transport activity enhances the relative amount of ATP produced by photosynthesis that can be used to fuel P-type ATPases or other energy-demanding processes during salt acclimation. However, increased involvement of PSI in cyclic electron transport activity lowers the electron flow from PSII that could result in its destabilization. As the respiratory chain is also situated at the thylakoid membrane in cyanobacteria, the terminal oxidase could also accept electrons originally coming from PSII and, therefore, prevent over-reduction of the thylakoid membrane system.

Besides P-type ATPases and respiration, other mechanisms might exist that alternatively or additionally could be involved in the energetization of ion transport via the establishment of a membrane potential. A protein, PxA, was described as essential for proton extrusion from cells of Synechocystis 6803 (Sonoda et al., 1998). Recently, it was also shown that the Mrp system is not only involved in ion transport but seems to participate in the energy metabolism of Nostoc (Anabaena) 7120 (Blanco-Rivero et al., 2009). Additionally, this system seems to be crucial for HCO\(_3\)\(^{-}\) uptake in cyanobacteria, as the mrp gene cluster is often located downstream from bicA or sbtA, both of which code for putative Na\(^{+}\)/HCO\(_3\)\(^{-}\) symporters. Probably, the Mrp system functioning as a Na\(^{+}\)/H\(^{+}\) antiporter maintains the Na\(^{+}\) gradient for the uptake of inorganic carbon, especially under alkaline conditions (Fukaya et al., 2009).
Whether cyanobacteria express a primary active sodium pump is still an open question. Ritchie (1992a) concluded from his bioenergetic studies on Na\(^+\) transport that, at least under alkaline conditions, a primary active transport is necessary, as under alkaline conditions the pH gradient at the cytoplasmic membrane is inverted, probably resulting in lower values of the proton motive force. There are a few reports on primary active Na\(^+\) transport in cyanobacteria (Brown et al., 1990) possibly involving Na\(^+\)-transporting ATPases or Na\(^+\)/K\(^+\) ATPases (Iwano, 1995). Such an idea is very attractive because in high-salt medium the Na\(^+\) gradient could be used as an energy source by establishing a \(\Delta V_{Na}^{+}\) across the cytoplasmic membrane (Brown et al., 1990). However, no directly active Na\(^+\)-pumping protein has yet been identified in cyanobacteria despite the many known complete genome sequences. This unclear situation resembles that in eukaryotic algae (Gimmler, 2000). Alternatively, the problems emerging from bioenergetic studies could be circumvented by assuming that the activity of Na\(^+\)-/H\(^+\) antiporters and P-type H\(^+\) ATPases is closely coupled. A colocalization of these proteins at the cytoplasmic membrane could be sufficient to maintain the membrane potential and thereby the proton motive force. Another candidate for the direct, active export of Na\(^+\) could be an ATP-dependent transporter of the ATP-binding cassette (ABC) type. In Bacillus subtilis, such a system has been identified to be involved in Na\(^+\) export (Cheng et al., 1997). Cyanobacterial genomes harbor many genes for ABC transporters, but the substrate specificity of most of them is unknown.

K\(^+\) content and transport

In contrast to Na\(^+\), the potassium ion is present in high amounts in control as well as salt-loaded cells. K\(^+\) is not only crucial for salt and/or turgor acclimation but is also involved in membrane energetics and the regulation of pH, enzyme activities, and gene expression (Wood, 1999; Gralla & Vargas, 2005; Ballal et al., 2007). Exact values for intracellular K\(^+\) concentrations are rare, because, in most cases, the concentrations are related to total cell volume. However, the problem of contamination and washing is much smaller in the case of K\(^+\) because its concentration outside the cell is usually much lower than inside the cell. Using NMR or radioactive isotopes, K\(^+\) levels of about 200 mM have been estimated for different cyanobacterial strains (Table 1), which is close to the known values in enterobacteria such as E. coli (Ballal et al., 2007). The concentration of K\(^+\) is slightly increased in long-term salt-acclimated cells, but this increase is clearly not sufficient to compensate for the increase in external ion concentration.

A different picture emerged when the kinetics of K\(^+\) concentration changes was measured after salt shock. These experiments showed a rapid accumulation of high K\(^+\) amounts, which followed the rapid Na\(^+\) influx and seemed to replace it almost completely (Reed et al., 1985b). In cells of Synechocystis 6714, the K\(^+\) uptake started a few minutes after the addition of 500 mM NaCl, peaked around 30–60 min, and gradually returned to levels similar to control cells after 24 h. Following the kinetics of K\(^+\) changes in salt-stressed cells of Synechocystis 6803 in more detail, it was recently shown that the rapid uptake of K\(^+\) after a few minutes is preceded by a short phase of K\(^+\) loss (Matsuda et al., 2004). This apparently outward diffusion of K\(^+\) parallels the phase of rapid Na\(^+\) entry, which is likely due to the opening of channels facilitating the influx of high amounts of Na\(^+\) and the efflux of K\(^+\) along their respective concentration gradients while the membrane potential is transiently collapsed (Reed et al., 1985a; Matsuda et al., 2004). The final reduction of K\(^+\) from long-term salt-stressed cells is accompanied by the massive accumulation of organic compatible solutes (Reed et al., 1985b). These changes in the intracellular solute pools resemble the well-studied situation in E. coli, in which a rapid K\(^+\) uptake (5 min) is similarly induced by salt shock, followed by a reduction in K\(^+\) and the accumulation of the compatible solute trehalose after longer times (60–120 min) at high salt concentrations (Dinnbier et al., 1988). In E. coli, K\(^+\), together with its organic counterion glutamate, is believed to act as compatible solute (at least K\(^+\) is much more compatible with cellular metabolism than Na\(^+\)) and to play a crucial regulatory role in the initiation of the subsequent acclimation processes (Wood, 1999; Gralla & Vargas, 2005). This regulatory function might also be relevant in the short-term salt acclimation of cyanobacteria, in which K\(^+\) accumulation represents an intermediary step to activate cellular metabolism before the salt-acclimated state is achieved, which is finally characterized by rather low inorganic ion concentrations, similar to control cells, and massive accumulation of compatible solutes.

The much higher concentration of intracellular K\(^+\) than extracellular K\(^+\) points to an active uptake of this important cation from the medium. Initially, kinetic measurements seemed to indicate that a Na\(^+\)/K\(^+\) exchange might be responsible for the observed monovalent cation relations. In animals, a primary active Na\(^+\)/K\(^+\) ATPase exists, which acts in such an exchange and in osmoregulation. There is one report indicating that a related activity might exist in cyanobacteria, as inhibitors of animal Na\(^+\)/K\(^+\) ATPases, such as ouabain, were found to inhibit ion transport in Nostoc strain M-14 (Iwano, 1995). However, these findings have not been supported by independent data, and analysis of the genomes of about 50 different cyanobacterial strains did not reveal any candidate gene for such an ATPase. In E. coli, three types of K\(^+\) transporters exist: the high affinity, ATP-dependent Kdp system and the low-affinity Trk and Kup systems (Wood, 1999). In the genome sequences of...
cyanobacteria, Kdp subunits, proteins related to Trk but named Ktr, and different putative K⁺ channels have been assigned as potential candidates for performing K⁺ uptake (Fig. 1). A major and sufficient role of K⁺ channels in potassium uptake has been demonstrated for the Gram-positive bacterium Corynebacterium glutamicum (Follmann et al., 2009).

The Kdp system initially received much attention, as this system is known from E. coli to be responsible for the immediate uptake of K⁺ after salt or osmotic shock, and it is characterized by the necessary affinity to cope with the low K⁺ concentration (Wood, 1999) characteristic of cyanobacterial media. The existence, function, and regulation of Kdp have been extensively studied by the Apte group in India. This work was recently reviewed by Ballal et al. (2007). It was found that all cyanobacteria possess the structural genes for a functional ATP-dependent K⁺ transport system consisting of the KdpABC subunits. The KdpA subunit is the K⁺ permease, the KdpB subunit is a typical P-type ATPase that provides the energy, and KdpC is involved in the assembly of the transport system. Interestingly, filamentous strains such as Nostoc (Anabaena) 7120 usually possess two operons for all structural kdp genes, designated kdp1 and kdp2, whereas unicellular freshwater strains have one operon, of the kdp2 type (Ballal et al., 2007). However, there are also many cyanobacteria without any kdp gene cluster. Besides Thermo-synechococcus elongatus BP1, all marine picoplanktonic strains of the genera Prochlorococcus and Synechococcus lack kdp genes in their completely known genome sequences (Cyanobase: http://genome.kazusa.or.jp/cyanobase/; Cyanorak: http://www.sb-roscoff.fr/Phyto/Genome_Cyanos/), which implies that the kdp operon has a low contribution to steady-state salt acclimation.

In E. coli, the kdp operon is induced under K⁺-limiting conditions (2 mM) and under osmotic and/or salt stress (Wood, 1999). The cyanobacterial kdp operons seem to be upregulated by low K⁺ concentrations but not by osmotic or salt stress (Alahari et al., 2001; Ballal et al., 2007). These data imply that the Kdp system is necessary for the import of K⁺ at extremely low external concentrations, but that the system plays no important role during salt acclimation of cyanobacteria. The latter assumption is supported by the finding that a kdpA mutant of Synechocystis 6803 did not show a reduced salt tolerance (Berry et al., 2003). Based on rather indirect evidence, the affinity of the Kdp system among cyanobacteria seems to be much higher than that reported for E. coli. This view is mainly based on the finding that a threshold of about 50 μM, i.e. 40 times lower than that in E. coli, has been found for the induction of kdp genes under K⁺-limiting conditions in Anabaena strains (Ballal et al., 2007). This finding could imply that the affinity of the functional Kdp system expressed is in the low μM or nM range, which corresponds to the much lower K⁺ content in the typical environment of cyanobacteria compared with that of E. coli.

The K⁺ and stress regulation of the E. coli kdp operon is performed via the KdpDE two-component system, which is found downstream of the structural genes (see Wood, 1999). The KdpD protein is a typical histidine kinase (Hik), found in the cytoplasmic membrane and believed to sense internal K⁺ and/or osmotic signals. KdpE is the associated response regulator (Rre), which is regulated by a phosphorelay from the autophosphorylated Hik and can bind to the promoter of the kdp operon for structural genes. The binding of KdpE represses kdpFABC expression under K⁺-replete conditions, whereas the release of KdpE at low K⁺ or the deletion of the kdpDE genes results in derepression of the kdp operon. Interestingly, in cyanobacteria, at first only a truncated KdpD protein was detected, which is encoded downstream of the structural genes in the kdp2 operon (Ballal et al., 2007). This truncated protein represents the N-terminal domain of the E. coli KdpD, whereas the transmembrane and Hik domains are missing. In an elegant study, the function of this truncated KdpD was verified by producing a chimeric protein of the E. coli Hik domain and the cyanobacterial N-terminal portion, which was called Ana-coi KdpD (Ballal et al., 2002). Indeed, the chimeric protein was able to complement a kdpD mutant of E. coli. In the currently available complete genome sequences, a two-gene operon is situated upstream from the kdp2 operons in Synechocystis 6803 and Nostoc (Anabaena) 7120 on the opposite DNA strand that encodes a typical two-component system with a Hik and a Rre (Ballal et al., 2007). It is very probable that this Hik represents the missing part of KdpD; if so, then a split KdpD rather than a truncated KdpD exists in cyanobacteria. In Synechocystis 6803, the Hik upstream of kdpA was designated Hik20 (Sll1590) and the associated protein Rre19 (Sll1592) (Cyanobase). The group of N. Murata, in Okazaki, Japan, generated a mutant library containing the complete set of Hik-defective strains for Synechocystis 6803. This mutant library was screened for changes in global gene expression in control as well as cold- or salt-stressed cells (Suzuki et al., 2000; Marin et al., 2003). The DNA microarray data indicated that the Hik20 protein is probably the Hik domain of KdpD involved in kdp regulation, as kdp expression is highly upregulated in the hik20 mutant under K⁺-replete conditions (Murata & Suzuki, 2006). These microarray experiments also supported the view that the Kdp system is of low importance for salt acclimation of Synechocystis 6803, as the kdp genes were not regulated by salt or osmotic stress, and the defect in Hik20 also did not change the salt tolerance or the expression of any salt-regulated gene (Kanesaki et al., 2002; Marin et al., 2003). However, the uptake of K⁺ via KdpABC might play a role under desiccation stress, as the kdp2 operon was clearly upregulated after drought stress in Nostoc (Anabaena) 7120.
and KtrE (missing in enterobacteria), respectively and the two small subunits KtrA (also found in enterobacteria) and KtrB, which forms the permease, and the two small subunits KtrA (also found in enterobacteria) and KtrE (missing in enterobacteria), respectively (Matsuda et al., 2004). Genes encoding similar proteins can be found in all known cyanobacterial genomes, including those of the marine picoplanktonic genera Synechococcus and Prochlorococcus (Cyanobase: http://genome.kazusa.or.jp/cyanobase/; Cyanorak: http://www.sb-roscoff.fr/Phyto/Genome_Cyanos/). The identification of this crucial K\textsuperscript{+} transport system was delayed because the gene slr1510 was initially annotated as ntpJ, which was thought to be a subunit of a Na\textsuperscript{+}-ATPase from enterobacteria, and not as KtrB. Therefore, the existence of the putative NtpJ subunit was taken as evidence for the existence of a primary active Na\textsuperscript{+} transport system. Accordingly, the salt sensitivity of ktrB mutants was initially attributed to a defective regulation of internal Na\textsuperscript{+} concentration (Shibata et al., 2002; Wang et al., 2002). Later on, it appeared that the ntpJ gene did not belong to the operon for the Na\textsuperscript{+}-ATPase in enterobacteria, but was involved instead in K\textsuperscript{+} transport (see discussion in Berry et al., 2003; Matsuda et al., 2004). Again, in these latter studies with Synechocystis 6803, it was found that the deletion of ktr genes resulted in a salt-sensitive phenotype, whereas a kdpA mutant could grow in high salt concentrations and that K\textsuperscript{+} transport, rather than Na\textsuperscript{+} transport, was affected. These results revealed that the Ktr system, rather than the Kdp system, is the main K\textsuperscript{+} import system in cyanobacteria. Another reason for the late attention to the Ktr system was that the related E. coli Trk system has a rather low affinity for K\textsuperscript{+} and was therefore believed to be unable to import this cation at low concentrations. However, the cyanobacterial Ktr system has a much higher affinity for K\textsuperscript{+}. Its $K_m$ was estimated to be around 60 $\mu$M, and therefore it is sufficient to catalyze K\textsuperscript{+} uptake in the typical, rather K\textsuperscript{+}-poor environment of cyanobacteria (Matsuda et al., 2004).

The view that Ktr functions only in K\textsuperscript{+} uptake is complicated by the fact that the Ktr system is also dependent on Na\textsuperscript{+} ions; i.e., high K\textsuperscript{+} transport rates were only observed when low mM concentrations of Na\textsuperscript{+} were present. Therefore, it was first assumed that this transporter might exchange K\textsuperscript{+} with Na\textsuperscript{+} or use the Na\textsuperscript{+} gradient to import K\textsuperscript{+}. However, expression of the Ktr system from Synechocystis 6803 in corresponding K\textsuperscript{+}-dependent E. coli mutants clearly revealed that the Ktr system is not able to transport Na\textsuperscript{+}. Most probably, the Na\textsuperscript{+} binding to the KtrB subunit induces a conformational change that subsequently stimulates K\textsuperscript{+} transport (Matsuda et al., 2004). The direct activation by Na\textsuperscript{+} binding also explains how the K\textsuperscript{+} transport activity is rapidly enhanced upon salt shock. Obviously, the external and/or internal Na\textsuperscript{+} concentration directly regulates the transport activity. In contrast, the expression of the Ktr system is not affected during the course of salt acclimation. Only ktrB was upregulated (by a factor of three) immediately after salt shock, but its expression soon returned to control levels (Marin et al., 2004). The driving force for K\textsuperscript{+} uptake by Ktr is probably the membrane potential. This view was recently supported in a bioenergetic study using different inhibitors affecting either the proton gradient or the membrane potential (Matsuda & Uozumi, 2006).

**Cl\textsuperscript{-} content and transport**

In contrast to the monovalent cations K\textsuperscript{+} and Na\textsuperscript{+}, much less is known about the transport of Cl\textsuperscript{-} in cyanobacteria, as is generally the case among bacteria with a few exceptions (e.g. Roeßler & Müller, 1998). The transport of Cl\textsuperscript{-} is much better characterized in eukaryotes (Jentsch, 2008). Similar to Na\textsuperscript{+}, Cl\textsuperscript{-} enters cyanobacterial cells in almost equimolar amounts after sudden salt shocks of $\geq 300$ mM NaCl; however, its export is slower than that of Na\textsuperscript{+} (Reed et al., 1985b). This slow export could be explained by the initial, rapid replacement of Na\textsuperscript{+} by high amounts of the monovalent cation K\textsuperscript{+}, for which Cl\textsuperscript{-} can also serve as a counter-ion. Subsequently, the amount of Cl\textsuperscript{-} becomes reduced in parallel with the export of K\textsuperscript{+}. In long-term salt-acclimated cells, the Cl\textsuperscript{-} content is similar to the Na\textsuperscript{+} content, pointing to its continuous export.

Bioenergetic studies indicated that cells in low-salt medium take up Cl\textsuperscript{-}, seemingly through an exchange with Na\textsuperscript{+} or by primary active transport using ATP (Ritchie, 1992b). The molecular mechanism for Cl\textsuperscript{-} export is still unknown among cyanobacteria. As it is negatively charged, an electrogenic export via Cl\textsuperscript{-} channels, such as in mammalian cells, is most likely. Because of sequence similarities to eukaryotic Cl\textsuperscript{-} channels, one gene (sll1864) was annotated as a putative Cl\textsuperscript{-} channel in Synechocystis 6803. Until now there has been no functional verification of this assumption. However, in DNA-microarray studies, it was found to be upregulated specifically by salt stress, whereas osmotic stress did not stimulate the expression of sll1864 (Kanesaki et al., 2002). Another candidate for a Cl\textsuperscript{-} transporter is the protein Slr0753. Mutation of sll0753 changed the Cl\textsuperscript{-} dependence of a PSII mutant of Synechocystis 6803. Similarity searches seem to indicate that it could be an outward-directed ion-transport system, probably specific for Cl\textsuperscript{-} (Kobayashi et al.,...
2006). This gene is also upregulated in cells of *Synechocystis* 6803 exposed to high NaCl concentrations (Marin *et al.*, 2004). In *E. coli*, the majority of Cl⁻ transport is done by H⁺–Cl⁻ exchange transporter and not via channels (Accardi & Miller, 2004). The closest *Synechocystis* 6803 homolog of that *E. coli* chloride transporter is encoded by *sll*0855.

**Synthesis of compatible solutes**

Compatible solutes are low-molecular mass organic compounds, which usually do not have a net charge and can be accumulated in high (molar) amounts without negatively interfering with cellular metabolism (Brown, 1976). The accumulation of compatible solutes in salt-stressed cells serves mainly to compensate for the difference in water potential that helps cells to take up water, establishing or maintaining turgor pressure. Additionally, numerous examples of the direct protection of macromolecules against denaturation induced by low water or high ionic concentrations have been shown. The action of compatible solutes is currently best explained by the water exclusion hypothesis (reviewed in Galinski, 1995; Record *et al.*, 1998b; Wood, 1999; Bremer & Krämer, 2000). According to this model, compatible solutes do not directly interact with macromolecules, such as proteins and/or membranes, during their protective action. Instead, they change the water structure, directing the remaining free water into the vicinity of the macromolecule surface, thereby retarding its hydration shell and preventing denaturation. Accordingly, the free-dissolved state of compatible solutes inside a cyanobacterial cell has been shown by NMR (Norton *et al.*, 1982). Among cyanobacteria, a correlation was found between the final salt tolerance limit and the major compatible solute (Fig. 2). Freshwater strains with low halotolerance accumulate sucrose and/or trehalose as their major compatible solute.

Moderately halotolerant (marine) strains are characterized by glucosylglycerol (GG) as their main compatible solute and sometimes glucosylglycerate (GGA) as a secondary compatible solute, whereas halophilic strains that are able to grow in saturated salt concentrations usually synthesize glycine betaine (GB) or glutamate betaine (Reed *et al.*, 1986a; Hagemann *et al.*, 1999b; Klähn *et al.*, 2010). Sucrose and trehalose are disaccharides known to be used as compatible solutes in plants, fungi, and/or heterotrophic bacteria. Much data are available on their versatile action in the acclimation not only to salt stress but also to other stresses, such as desiccation, cold, or heat. In contrast to other compatible solutes, it is assumed that sucrose and trehalose can directly interact with macromolecules. The steric organization of the hydroxyl groups of these soluble carbohydrates allows them contact with the head groups of glycerolipids. This enables sucrose and trehalose to replace at least part of the water in the hydration shells of membranes (see Potts, 2004).

Among cyanobacteria, the ability to synthesize sucrose seems to be universal, as at least one sucrose-synthesizing enzyme is encoded in almost all of the known genome sequences. The importance of sucrose synthesis for salt acclimation was first recognized by the stress-induced accumulation of sucrose in *Nostoc muscorum* strain 7119 (Blumwald & Tel-Or, 1982), *Synechococcus* 6301 (Blumwald *et al.*, 1983a), and *Anabaena variabilis* strain Lefevre 305 (Erdmann, 1983). The screening of many cyanobacterial strains revealed that sucrose is accumulated under high-salt conditions by at least 60 different strains (Supporting Information, Table S1). This large dataset supports the general trend...
that strains accumulating sucrose as the main compatible solute show a rather low salt tolerance and are often freshwater isolates (Reed et al., 1984b). As for the preferred accumulation of other compatible solutes, there is no correlation with any taxonomic unit; instead, sucrose-accumulating strains form a functional group of slightly salt-tolerant strains. The only exceptions are Nostoc and Anabaena, as all strains of these genera accumulate sucrose as the only significant organic-compatible solute under high-salt conditions. As in other photosynthetic organisms, such as plants, sucrose is part of the basic carbon metabolism of cyanobacterial cells. In diazotrophic cyanobacteria-forming heterocysts, sucrose is donated by the vegetative cells to the \( \text{N}_2 \)-fixing heterocysts, where it serves as the carbon skeleton for amino acid synthesis (Wolk et al., 1994). The participation of sucrose in primary metabolism might be a reason for restricting its accumulation to lower salt concentrations not sufficient to mediate higher levels of salt resistance.

In addition to the large number of strains with low salt tolerance that rely purely on sucrose accumulation, sucrose was found as a minor or transient part of the total compatible solute pool in strains accumulating GG, GGA, and/or GB, which help them to attain higher levels of salt resistance (see Table S1). A transient increase in sucrose is probably common in cyanobacterial strains upon exposure to increased salinities. Some interesting regulatory features have been shown for strains accumulating both sucrose and GG. In Synechocystis 6714 and 6803, the sucrose pool increased initially at higher rates than GG; however, the sucrose level becomes reduced in completely salt-acclimated cells characterized by the dominant solute GG (Reed et al., 1985b; Marin et al., 2004; Desplats et al., 2005). The growth temperature also has an impact on the proportions of GG and sucrose in cyanobacteria. An increase in the sucrose/GG ratio at elevated temperatures was observed in salt-treated cells of Synechocystis 6714 (Warr et al., 1985b). Similar trends have been reported for salt-loaded cells of eukaryotic microalgae; for example, Dunaliella tertiolecta showed a reduced content of its main compatible solute glyceral at the expense of higher sucrose concentrations at higher temperatures (Müller & Wegmann, 1978). The molecular basis for this shift is not clear. Sucrose is probably able to directly support membrane stability at higher temperatures, as it has been shown that sucrose protects model membranes \textit{in vitro} much better than GG or GB (Hinch & Hagemann, 2004). Accordingly, the expression of the Synechocystis \textit{sps}A gene for sucrose biosynthesis in \textit{E. coli} resulted in sucrose accumulation and increased desiccation tolerance in the recombinant cells (Billi et al., 2000). Evidence for a more general protective role of sucrose in cyanobacterial cells was obtained using a sucrose-synthesis deficient mutant of Synechocystis 6803. While such mutants did not show a decreased salt tolerance because of the intact GG accumula-

tion (Hagemann & Marin, 1999), cells of the sucrose-deficient strain revealed a clear defect after the transfer of stationary phase cells to fresh salt medium compared with wild type (Desplats et al., 2005). It is well known from other bacteria, such as \textit{E. coli} (Hengge-Aronis, 1996), that the transition of cells into stationary phase is accompanied by increased resistance to adverse conditions, where compatible solutes, such as trehalose in the case of \textit{E. coli}, act as stabilizers. The reduced resistance of sucrose-deficient cells of Synechocystis 6803 can, therefore, be taken as an indication of a similar role of sucrose among cyanobacteria. The frequently occurring accumulation of sucrose as a secondary or tertiary compound may accordingly be related to its general protective role, rather than to a salt-specific response.

The salt-induced sucrose biosynthesis in cyanobacteria is achieved by sucrose-phosphate synthase (SPS, EC 2.4.1.14), which produces the intermediate sucrose-phosphate from UDP-glucose and fructose-6-phosphate (Fig. 1). Sucrose-6-phosphate is subsequently hydrolyzed to sucrose by sucrose-phosphate phosphatase (SPP, EC 3.1.3.24). The combined action of SPS and SPP was shown in crude enzyme preparations from Synechocystis 6803 with biochemically purified native enzymes from \textit{Anabaena} 7119 and with recombinant enzymes obtained after overexpression of the corresponding genes from Synechocystis 6803 in \textit{E. coli} (Porchia & Salerno, 1996; Curatti et al., 1998; Hagemann & Marin, 1999; Lunn et al., 1999). In addition to UDP-glucose, the cyanobacterial SPS can use ADP-, GTP-, and CTP-glucose as glucosyl donors. Because of its close similarity to previously characterized plant \textit{sps} genes, the first cyanobacterial SPS gene, \textit{sps}A, was annotated in the genome of Synechocystis 6803 (Kaneko et al., 1996) and functionally confirmed by enzyme activity measurements of the purified recombinant protein as well as by mutation of the corresponding gene, abolishing sucrose synthesis (Curatti et al., 1998; Hagemann & Marin, 1999; Lunn et al., 1999). As found before for plant SPS proteins, the \textit{sps}A gene of Synechocystis 6803 encodes a large protein of about 80 kDa with an N-terminal domain containing all the features required for SPS activity and a C-terminal domain of about 20 kDa with similarities to SPP. Despite its structural similarity to the combined SPS/SPP proteins from plants, the SPS from Synechocystis 6803 showed only SPS activity (Lunn et al., 1999). The gene encoding the protein with cooperating SPP activity was identified later by similarity searches with SPP sequences from higher plants (Lunn, 2002). Recently, the crystal structure of the Synechocystis 6803 SPP has been solved, providing an explanation of the process of sucrose-phosphate dephosphorylation and indicating that it is very likely that the catalytically inactive SPP-like C-terminal domain of SpSA still is able to bind sucrose-phosphate (Fieulaine et al., 2005). In nearly all of the presently available cyanobacterial genomes, including the reduced genomes from marine...
picoplanktonic strains (only exception is Microcystis strain NIES-843), at least one and often two genes for SPS are annotated (Scanlan et al., 2009).

The expression of spsA is transiently increased after salt shock, which corresponds well to the transient sucrose accumulation in Synechocystis 6803 (Marin et al., 2004). Increased SPS activity could also be measured in crude extracts of salt-acclimated cells of Synechococcus 6301 and Nostoc (Anabaena) 7120, which accumulate sucrose as the sole compatible solute. Moreover, SPS activity could be directly stimulated in crude extracts from Synechocystis 6301 by the addition of NaCl to the enzyme assays, whereas this was not the case for the Anabaena enzyme (Hagemann & Marin, 1999). Another pathway of sucrose biosynthesis depends on the action of sucrose synthase (SuSy, EC 2.4.1.13). This enzyme catalyzes the reversible conversion of UDP-glucose and D-fructose to sucrose and UDP or vice versa. SuSy activity was detected in Anabaena 7119 (Porchia et al., 1999). Subsequently, Salerno’s group from Argentina cloned two SuSy genes from Anabaena 7119. Functional characterization of mutants revealed that these enzymes are involved in diazotrophic growth, where at least SusA acts in sucrose breakdown, based on the finding that, in cells of the susA mutant, sucrose was accumulated and N2 fixation was abolished (Curatti et al., 2002). The preferred action of SuSy in the process of N2 fixation, particularly in heterocyst-forming cyanobacterial strains, but not in salt acclimation is supported by the strain-specific occurrence of the SuSy genes, which seem to be restricted to heterocyst-forming cyanobacteria and are absent in unicellular strains, such as Synechocystis 6803 or Synechococcus 6301.

Compared with sucrose, much less attention has been paid to trehalose accumulation in salt-stressed cyanobacteria. Trehalose accumulation was first demonstrated in Rivularia atra collected from the tidal zone, where it is exposed to changing salt and water availability (Reed & Stewart, 1983). Since then, trehalose accumulation has been detected in > 40 strains, when cultivated in NaCl-enriched medium, but trehalose is the sole or dominant compatible solute in only 20 of them (Table S1). Interestingly, trehalose-accumulating cyanobacteria are mostly filamentous strains isolated from terrestrial habitats or from freshwater and coastal areas, where they often form mats or aggregates. Comparable to sucrose, the accumulation of the carbohydrate trehalose results mostly in a rather low degree of salt resistance, but its occurrence as a minor solute in GG- or GB-accumulating strains may provide a more general stress tolerance, as discussed above for sucrose. Trehalose is known to have superior protective features for membranes (e.g. Hincha & Hagemann, 2004). It is worth mentioning that trehalose accumulation is a common feature for desiccation-tolerant cyanobacteria such as Nostoc or Sytonema sp. (Hershkovitz et al., 1991; Page-Sharp et al., 1999; Potts, 2004).

The mode of trehalose synthesis in cyanobacteria is not completely known. Trehalose is the major organic compatible solute in E. coli when it is exposed to salt stress in minimal medium. There, it is formed by the cooperative action of trehalose-phosphate synthase (TPS), using glucose-6-phosphate and UDP-glucose to generate the intermediate trehalose-6-phosphate, which is subsequently dephosphorylated to trehalose by trehalose-6-phosphate phosphatase (TPP). The two reactions are catalyzed by separate proteins, encoded by the genes otsA and otsB for TPS and TPP, respectively. These genes form the salt and osmotic stress-induced otsBA operon in the E. coli genome (Strom & Kaasen, 1993). A comparable pathway for trehalose synthesis has been found in yeast and plants, but in these organisms TPS and TPP are often fused into a large protein, comparable to the situation of SPS and SPP activities described above. At least three different routes for trehalose biosynthesis were characterized in the Gram-positive bacterium C. glutamicum (Wolf et al., 2003). Besides the TPS/TPP pathway, the enzyme TreS was found to convert maltose into trehalose (TreS pathway). The maltoligosyl-trehalose-synthase (Mts or TreY) and -hydrolase (Mth or TreZ) enzymes are able to convert high molecular mass precursors, such as glycogen or starch, into trehalose (TreY/TreZ pathway; for review see Empadinhas & da Costa, 2008a). Corresponding trehalose-synthesizing enzyme activities were detected in a trehalose-accumulating Sytonema isolate. In crude extracts of Sytonema sp., the presence of TPS and TPP activities could be shown. TPS showed higher activities with ADP-glucose than with UDP-glucose. While these enzyme activities were rather low in control cells, an increase was found in salt-shocked cells in parallel with active trehalose synthesis (Page-Sharp et al., 1999). These results point to a trehalose biosynthetic pathway comparable to that of E. coli, which seems to be mainly regulated at the gene expression level. Unfortunately, the gene sequences for the TPS and/or TPP in Sytonema are not yet known. Only the genome of Crocosphaera watsonii WH8501 harbors genes similar to otsA and otsB from E. coli (http://genome.jgi-psf.org/crowa/crowa.home.html). Their functional verification would provide conclusive evidence that this pathway truly exists in at least some trehalose-accumulating cyanobacterium.

The alternative TreY/TreZ pathway for trehalose biosynthesis was verified in the model strain Nostoc (Anabaena) 7120. Using a genome-wide DNA microarray, many desiccation-induced genes were identified in this strain. Among them, the authors found upregulation of a gene cluster encoding proteins with similarities to TreY (Mts) and TreZ (Mth) that are possibly involved in the synthesis of trehalose by transglucosidase reactions starting from glycogen (Kato et al., 2004). The expected capacity for trehalose biosynthesis was verified in a subsequent analysis using recombinant
proteins obtained by overexpression of the Nostoc genes in E. coli. Moreover, corresponding mutants were unable to synthesize the rather small amounts of trehalose typically observed transiently after the onset of desiccation stress in cells of Nostoc (Anabaena) 7120. Furthermore, the treH gene encoding a trehalase was found to be linked to these trehalose synthesis genes (Higo et al., 2006). Similar genes were found in the genomes of the closely related strains A. variabilis and Nostoc punctiforme. Recently, the drought-and salt-induced synthesis of trehalose was confirmed in N. punctiforme strain IAM M-15 and shown to also be catalyzed by the TreY/TreZ pathway that seemed to be regulated at a biochemical level. The mRNA levels of all the tre genes were not changed after exposure to environmental stresses; however, the synthesis enzymes were activated by increasing salt concentrations was found, whereas the activity of trehalase was inhibited under these conditions (Yoshida & Sakamoto, 2009). At the moment it is not clear whether the accumulation of trehalose in salt- or desiccation-stressed cyanobacteria is based generally on the TreY/TreZ pathway or also on the more classical OtsAB-like pathway. To answer this question, further molecular investigation or genome sequencing of a truly desiccation-tolerant strain accumulating high amounts of trehalose is necessary.

**Glucosylglycerol**

GG [\(\alpha-D\)-glucopyranosyl-(1 → 2)-glycerol] represents another typical compatible solute of cyanobacteria. Actually, it was the first osmoprotective compound identified in a cyanobacterial strain. Applying the \(^{13}\)C-NMR-technique to intact cells of the marine cyanobacterium Synechococcus N100, Borowitzka et al. (1980) noticed prominent NMR signals that correlated in intensity with the salt content of the growth medium. The substance was identified to be identical to GG, which had previously been described as a natural component of a cyanobacterial extract (Kollman et al., 1979). So far, the salt-induced accumulation of GG as a main compatible solute has been reported for about 60 different strains of many different cyanobacterial genera (Table S1). Often, these strains also accumulate smaller amounts of other compatible solutes, such as sucrose and/or trehalose. GG was also found as a minor compound in GB-accumulating cyanobacterial strains.

On average, strains with GG as their main compatible solute are able to tolerate salinities at least twice as high as that of seawater (equivalent to 7% NaCl or 1.2 M NaCl) but can also propagate in mineral medium without salt supplementation (Fig. 2). This specific range of salt resistance led originally to the hypothesis that GG accumulation is characteristic of cyanobacterial strains from marine habitats (Mackay et al., 1983). However, GG was also detected in many cyanobacteria originating from freshwater (Reed et al., 1984b), overturning the assumption that only marine cyanobacteria accumulate GG. Nevertheless, GG accumulation provides a characteristic degree of salt resistance significantly higher than that of sucrose- and/or trehalose-accumulating strains. This finding led to the definition of the functional group of moderately halotolerant cyanobacterial strains that is characterized by GG as the dominant compatible solute (Reed et al., 1986a; Hagemann et al., 1999b).

Initially, it was thought that GG synthesis and accumulation is restricted to cyanobacteria, as, for > 10 years, GG was only reported as a compatible solute among these organisms. However, Bianchi et al. (1993) found accumulation of GG and steric isomers in drought-stressed leaves of the resurrection plant Myrothamnus flabellifolia. Since then, GG accumulation has been found in many heterotrophic bacteria, such as different Pseudomonas and Stenotrophomonas strains (Pocard et al., 1994; Mikkat et al., 2000a; Roder et al., 2005; Hagemann et al., 2008). Later, GG was also identified in traditional Japanese foods, such as sake, mirin, and miso ( Takenaka et al., 2000). These foods are produced by a fermentative process employing the koji mold, Aspergillus oryzae, which is probably responsible for the synthesis of GG from rice starch.

Cells of cyanobacteria shown to produce GG are virtually free of this compatible solute when grown in low-salt mineral medium. GG synthesis starts immediately after the addition of NaCl, without any lag phase. After several hours, a stress-proportional steady-state level is reached (Reed & Stewart, 1985; Reed et al., 1985b; Warr et al., 1985a; Hagemann et al., 1987). This level is mainly determined by the regulation of GG synthesis, as no significant GG turnover was observed in salt-acclimated cells. GG synthesis depends on photosynthetic carbon fixation. In salt-shocked cells, GG presents one of the most strongly labeled substances in \(^{14}\)C-labeling-experiments (Erdmann, 1983; Hagemann et al., 1987). Using \(^{13}\)C-labeled cells, the synthesis of GG from accumulated glycogen could be also shown (Tel-Or et al., 1986; MacKay & Norton, 1987). In these experiments with Agmenellum quadruplicatum (syn. Synechococcus 7002), no GG synthesis was observed in dark-incubated cells, whereas low rates of GG accumulation could be measured when stressing cells of Microcystis firma strain Gromov 398 with salt in the darkness. However, the GG levels were never sufficient to sustain growth of salt-treated cells in darkness (Erdmann et al., 1989). Hypo-osmotic shocks led to a rapid disappearance of GG from previously salt-acclimated cells. When the strength of the salt down-shock exceeded a certain threshold (about 300 mM NaCl) almost all of the GG was released into the medium (Reed et al., 1986b; Fulda et al., 1990). It is thought that mechanosensitive channels (MscL and MscS, Fig. 1) open under hypo-osmotic stress to prevent the bursting of cells when water is moving into the hypo-osmotically shocked cells along the water potential.
Another striking feature of this enzymatic system. As with observed in vitro demands (Hagemann GG in the light to meet the external salinity and growth of cyanobacteria, which usually grow actively and produce new plastes in anaerobic, salt-treated cells of Synechocystis. However, the described examples of GG breakdown represent rather exceptional situations for photoautotrophic cyanobacteria, which usually grow actively and produce new GG in the light to meet the external salinity and growth demands (Hagemann et al., 1987; Marin et al., 2002).

The biosynthesis of GG in cyanobacteria is performed by the typical pathway for the synthesis of glucosides (Fig. 1), using ADP-glucose and glycerol-3-phosphate (G3P) as precursors (Hagemann & Erdmann, 1994). It should be mentioned that GG synthesis in the cyanobacterial strain Synechocystis 6803 was found to be strictly dependent on ADP-glucose, whereas for sucrose and trehalose biosynthesis, UDP- and ADP-glucose could be used, respectively. The first step in the GG biosynthesis pathway is catalyzed by GG-phosphate synthase (GgpS, EC 2.4.1.213), leading to formation of the intermediate GG-phosphate (GGP) and ADP. Afterwards, GGP is dephosphorylated by GG-phosphate phosphatase (GgpP, EC 3.1.3.69), a sugar-phosphate phosphatase very specific for GG-phosphate. A mutant of Synechocystis 6803 defective in this enzyme accumulated GG-phosphate, so, obviously, no other sugar-phosphate phosphatase could take over the dephosphorylation of this intermediate sufficiently (Hagemann et al., 1997b).

The salt dependence of GG synthesis in vitro represents another striking feature of this enzymatic system. As with intact cells of Synechocystis 6803, GG synthesis was only observed in vitro with salt-supplemented buffers, while the addition of noncharged organic compounds, such as sucrose, was only effective to activate GG synthesis in vivo but not in vitro (Hagemann & Erdmann, 1994). However, it should be noted that osmotic shocks by permeable organic compounds, such as the widely used sorbitol, did not activate GG biosynthesis. Accordingly, a rather low tolerance of Synechocystis 6803 to sorbitol treatments was observed (Marin et al., 2006). In crude protein extracts, GgpS as well as GgpP became activated when about 100 mM NaCl was added. Moreover, a remarkable tolerance to high levels of NaCl (up to 1 M), inhibitory for most normal enzymes, was shown in the in vitro assay system (Hagemann et al., 1996). Other ions besides NaCl were capable of activating the enzyme activities according to the Hofmeister series, in which the ions with the highest chaotropic features were most efficient (Schoor et al., 1999). This salt-stimulation of GgpS and GgpP completely explains the initial activation of GG synthesis in salt-shocked cyanobacterial cells characterized by transiently high ion contents. The inorganic ions seem to remove an inactivating molecule or modification from the GG-synthesizing enzymes, as pure enzymes obtained after biochemical purification or overexpression of the corresponding cyanobacterial genes in E. coli already showed activity in low-salt buffers. However, the pure enzymes could be inactivated by mixing with total extracts from low-salt-grown cells of Synechocystis 6803. This salt-dependent activation and inactivation was found to be completely reversible (Hagemann et al., 2001). The underlying biochemical mechanism remains to be characterized. Preliminary data seem to indicate that protein phosphorylation, regulation by phosphorylated metabolites, and/or proteolytic processes might be involved (Hagemann et al., 1993, 2001; Stirnberg et al., 2007).

The genes for GG synthesis were identified in the characterization of salt-sensitive mutants of Synechocystis 6803 (Jeanjean et al., 1990; Hagemann & Zuther, 1992). Initially, the stpA gene [salt tolerance protein A (StpA)] was cloned from a Synechocystis mutant, which showed the lowest level of remaining salt tolerance (about 0.2 M NaCl). This mutant showed a pleiotropic phenotype and therefore the StpA protein was initially thought to be a regulator necessary in the basic salt acclimation processes (Onana et al., 1994). Later, the same gene was identified as defective in another salt-sensitive Synechocystis mutant accumulating the intermediate GG-phosphate. This finding made the stpA gene a very promising candidate for the GgpP-encoding gene. Overexpression of this gene in E. coli and demonstration of GgpP activity with the purified recombinant enzyme supported this assumption (Hagemann et al., 1997b). Obviously, the GgpP (StpA) represents a new class of sugar-phosphate phosphatases that occurs specifically in GG-accumulating cyanobacteria. The only exceptions are Prochlorococcus strains that also harbor genes very similar to the ggpP but do not have a ggpS and do not accumulate GG (Klähn et al., 2010).

The gene for the first enzyme in the GG synthesis pathway, GgpS, was identified using another salt-sensitive mutant of Synechocystis that totally lacked GG (Marin et al., 1998). The affected gene encoded a protein showing considerable similarities to the trehalose-phosphate synthase OtsA from E. coli. The overexpression of this gene in E. coli yielded a protein with specific GgpS activity. Therefore, the gene sll1566 of Synechocystis 6803 (Kaneko et al., 1996)
clearly represents the GgpS-encoding gene. Subsequently, a second ggpS gene was cloned and functionally characterized from the marine strain *Synechococcus* 7002 (Engelbrecht *et al.*, 1999). Cyanobacterial mutants with inactivated ggpS genes showed a salt-sensitive phenotype, despite the fact that an increased content of the secondary compatible solute sucrose was detected. However, the final sucrose content was not sufficient to compensate for the higher salt concentrations in the growth medium, leading to a reduction of salt tolerance < 0.3 M NaCl (Marin *et al.*, 1998; Engelbrecht *et al.*, 1999). In a *Synechocystis* 6803 mutant where ADP-glucose pyrophosphorylase (Agp), the enzyme necessary to synthesize the GG precursor ADP-glucose, was knocked out, the complete absence of GG did not result in salt sensitivity. Instead of GG, this *agp* mutant accumulated very high levels of sucrose. Not only GG synthesis but also glycogen synthesis was completely blocked, which probably led to an increased UDP-glucose pool sufficiently high to ensure massive sucrose accumulation (Miao *et al.*, 2003). However, a regulatory role for UDP- and/or ADP-glucose cannot be ruled out. It was reported that the amount of UDP-glucose in the cell mediates the expression of many stress-regulated genes, including the *otsBA* operon in *E. coli* (Böhringer *et al.*, 1995).

Genes encoding proteins very similar to the GgpS from *Synechocystis* 6803 are present in the genomes of all strains known to produce GG, including marine, picopelagial isolates of the genus *Synechococcus* (Scanlan *et al.*, 2009; Klähn *et al.*, 2010). In phylogenetic analyses, all cyanobacterial GgpS proteins form two closely linked clades that are separated from the recently identified GgpS sequences of heterotrophic bacteria (Fig. 3). The GgpS proteins from picopelagial cyanobacteria are found to be closely related to the different GgpS proteins from ‘classical’ cyanobacteria, including *Synechocystis* 6803. Interestingly, two different types of GgpS proteins were found among heterotrophic bacteria. One type of protein is very similar to that from *Synechocystis* 6803 and clusters close to or within the clade of ‘classical’ cyanobacterial GgpS sequences (Fig. 3). A second cluster of enzymes from heterotrophic bacteria comprises proteins of the glucosylglycerol-phosphate phosphatases/synthases (GgpPS) type. The GgpPS proteins are fusion proteins, in which the GgpS is found at the C-terminal end and the GgpP at the N-terminal end. These proteins have recently been proven capable of producing GG from ADP-glucose and G3P as a single protein in heterotrophic bacteria, such as *Stenotrophomonas rhizophila* or *Azotobacter vinelandii* (Hagemann *et al.*, 2008).

**Glycine betaine**

The quaternary ammonium compound GB (*N*,*N*,*N*-trimethylammonioacetate) has long been described as a characteristic compatible solute in heterotrophic bacteria and plants conferring high levels of salt tolerance. The first cyanobacterial strain proven to contain GB as a major compatible solute was *Synechocystis* sp. DUN52 that was isolated from mats in intertidal coastal waters of Kuwait (Mohammad *et al.*, 1983). Later, it was found to be the dominant compatible solute in about 15 different strains (Table S1) all characterized by high salt tolerance levels. Among these strains, many true hypersaline strains were found that could grow in medium nearly saturated in NaCl but not in freshwater medium without supplementation of a minimal NaCl amount (Mackay *et al.*, 1984; Reed *et al.*, 1984a; Gabbay-Azaria *et al.*, 1988; Lu *et al.*, 2006). Besides GB, these strains usually accumulate sucrose, trehalose, and/or GG as additional compatible solutes at lower concentrations.

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**Fig. 3.** Phylogenetic tree displaying different types of GgpS and GgpPS from cyanobacteria and heterotrophic bacteria. The trehalose-synthesis enzyme OtsA served as an outgroup. Bootstrap values are indicated.

<table>
<thead>
<tr>
<th>GgpPS</th>
<th>GgpPS heterotrophic bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>ggpPS Srhiz</td>
<td>ggpPS Xcampa</td>
</tr>
<tr>
<td>ggpPS Marinoa</td>
<td>ggpPS Azooar</td>
</tr>
<tr>
<td>ggpPS Avine</td>
<td>ggpPS Pseuud</td>
</tr>
<tr>
<td>ggpPS Pmend</td>
<td>ggpPS Dhib</td>
</tr>
<tr>
<td>ggpPS Roseo</td>
<td>ggpPS Rhodob</td>
</tr>
<tr>
<td>ggpPS Sdelt</td>
<td>ggpPS Tdel</td>
</tr>
<tr>
<td>ggpPS Pberm</td>
<td>ggpPS Syn6803</td>
</tr>
<tr>
<td>ggpPS Syn7002</td>
<td>ggpPS Cyanobacter</td>
</tr>
<tr>
<td>ggpPS Acaryochioris</td>
<td>ggpPS Acaryochioris</td>
</tr>
<tr>
<td>ggpPS Microcoles</td>
<td>ggpPS SynWH7003</td>
</tr>
<tr>
<td>ggpPS SynRCC307</td>
<td>ggpPS SynWH7005</td>
</tr>
<tr>
<td>ggpPS SynRS9517</td>
<td>ggpPS SynWH7005</td>
</tr>
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<td>ggpPS SynRS9516</td>
<td>ggpPS SynWH7003</td>
</tr>
<tr>
<td>ggpPS SynW1281</td>
<td>ggpPS SynWH7005</td>
</tr>
<tr>
<td>ggpPS SynCC9605</td>
<td>ggpPS SynBL107</td>
</tr>
<tr>
<td>ggpPS SynCC9902</td>
<td></td>
</tr>
</tbody>
</table>

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In heterotrophic bacteria and in plants, GB is usually synthesized by a two-step oxidation starting from choline via the toxic intermediate betaine aldehyde (reviewed in Bremer & Krämer, 2000). In the case of *E. coli*, the first step is catalyzed by BetA, an FAD-containing choline dehydrogenase. The conversion of the toxic intermediate is catalyzed by betaine aldehyde dehydrogenases, such as BetB in *E. coli* (Bremer & Krämer, 2000). There are also bacteria that have an enzyme capable of converting choline into GB by a one-step reaction in which a choline monoxygenase produces GB and the toxic byproduct H2O2 (Sakamoto & Murata, 2001). However, it is necessary to note that most of the heterotrophic bacteria rely on the uptake of the GB precursor choline from the environment for salt-induced GB synthesis, whereas plants and cyanobacteria are able to synthesize GB de novo.

Among cyanobacteria, GB biosynthesis has been best investigated in the hypersaline strain *A. halophytica* [syn. *Synechococcus* sp. strain Pasteur culture collection (PCC) 7418]. Initially, experiments were conducted by feeding potential 14C-labeled precursors to salt-loaded cells of *A. halophytica* (Incharoensakdi & Wutipradikul, 1999). The results indicated that there may be more than one route leading to GB in cyanobacteria. The addition of 14C-choline resulted in the synthesis of labeled GB. As there was also evidence for the labeling of the intermediate betaine aldehyde, these results pointed to a classical two-step oxidation pathway for GB synthesis in cyanobacteria. Accordingly, the activities of choline as well as betaine aldehyde dehydrogenase were detected in crude extracts of this cyanobacterial strain, and these activities were clearly enhanced in salt-acclimated cells accumulating higher GB levels. However, GB labeling was also obtained when salt-stressed cells of *A. halophytica* were incubated with 14C-ethanolamine, a precursor for choline biosynthesis, or with 14C-glycine. The conversion of labeled glycine into GB was used as an indication of a direct methylation pathway starting from glycine (Incharoensakdi & Wutipradikul, 1999), which had previously been shown to occur in a phototrophic bacterium (Trüper & Galinski, 1990).

The three-step methylation of glycine as a main pathway for GB synthesis among cyanobacteria was recently verified by Takabe’s group in Nagoya, Japan. Using *A. halophytica* as a model, this group was able to clone two genes that encode enzymes capable of methylating glycine and the first intermediate, sarcosine (glycine/sarcosine-<i>N</i>-methyltransferase, GSMT), or the other intermediate, dimethylglycine (dimethylglycine-<i>N</i>-methyltransferase, DMT). GSMT- and DMT-encoding genes were identified using information on the sequences of similar genes from heterotrophic bacteria. The biochemical features of these enzymes were verified using purified recombinant enzymes after overexpression in *E. coli* (Waditee et al., 2003). These enzymes showed a remarkably low feedback inhibition by the end product GB at concentrations of up to 2 M. Moreover, S-adenosyl methionine (SAM) was shown to be the methyl-group donor for GSMT and DMT. When the C1-group is removed from SAM, S-adenosyl homocysteine is produced. This byproduct is known to inhibit the two-step oxidative pathway for GB synthesis, but it interferes hardly at all with the activities of GSMT and DMT. Finally, both enzyme activities and the amounts of the corresponding proteins increased in salt-stressed cells of *A. halophytica*. The very comprehensive study of Waditee et al. (2003) indicates that cyanobacteria likely synthesize GB exclusively by the direct methylation pathway, starting from glycine. Unfortunately, there is no report of a specific GSMT and/or DMT mutant of *A. halophytica*. The complete absence of GB in such an *A. halophytica* mutant would unequivocally rule out the existence of other GB pathways. Recently, the GSMT and DMT pathway for GB synthesis was shown to also occur in the marine picoplanktonic strain *Synechococcus* WH8102 (Lu et al., 2006). Besides the genes for GSMT and DMT, no genes for enzymes known to be able to synthesize GB by another pathway are present in the complete genome sequence of *Synechococcus* WH8102.

The ability to synthesize choline as a precursor for two-step biosynthesis is probably limiting in cyanobacteria, as in heterotrophic bacteria. Two groups reported on the transfer of GB-synthesizing enzymes from heterotrophic bacteria into the freshwater strains *Synechococcus* 6301 or 7942. Despite the transfer of genes encoding enzymes involved in the two-step or one-step oxidation pathway, only very low amounts of GB were synthesized in the recombinant strains. Only after supplementation of the medium by choline was a slightly higher GB accumulation and a slightly improved salt tolerance observed (Deshnium et al., 1995; Nomura et al., 1995). In contrast, the transfer of genes for the three-step methylation of glycine resulted in much higher GB synthesis and improvement of salt resistance in the freshwater strain *Synechococcus* 7942 as well as in the model plant *A. thaliana* (Waditee et al., 2005).

Only very limited attention was drawn to the fate of GB in cells after hypo-osmotic shocks. Incharoensakdi & Waditee (2000) provided some evidence for the breakdown of GB by betaine-homocysteine methyltransferase rather than its release into the medium with cells of *A. halophytica*, which were transferred from medium containing 2 M NaCl into medium containing only 0.5 M NaCl. On the contrary, cells preloaded with 14C-labeled GB released this compound into the medium after a hypo-osmotic down-shock (Moore et al., 1987), which indicates that both release and degradation seem to occur when GB-accumulating cyanobacteria are challenged by hypo-osmotic shocks.

**Other compatible solutes**

The three cyanobacterial salt tolerance groups, freshwater strains, moderately halotolerant strains and hypersaline
strains, are characterized by their typical major compatible solutes, sucrose and trehalose, GG, and GB, respectively (Fig. 2). However, there are reports of other organic compatible solutes in salt-stressed cells of cyanobacteria. Besides GB, the quaternary ammonia compound glutamate betaine has been shown in two hypersaline *Calothrix* strains (Mackay et al., 1984, see Table S1). Obviously, this compound is functionally equivalent to GB. Unfortunately, no further investigations regarding its biosynthesis have been reported as yet.

Proline is another compatible solute often reported in salt-stressed cells of heterotrophic bacteria and plants. Unlike most other compatible solutes, proline and sucrose are used in primary metabolism. The action of proline as a potential compatible solute was first shown for the cyanobacterium *N. muscorum* by the isolation of mutants defective in proline breakdown by proline oxidase or after supplementation of the growth medium by proline and its subsequent uptake. The increased proline levels improved the salt resistance of *N. muscorum* (Singh et al., 1996). Proline accumulation was also shown for *Synechococcus* 7418 (*A. halophytica*), which mainly accumulates GB. Proline accumulation preceded the synthesis of GB in salt-stressed cells. Proline is probably required to (re)activate salt-affected enzymes, including those for GB synthesis. In completely salt-acclimated cells, high steady-state levels of GB were found, whereas proline concentrations were found to be reduced to low basal levels without any osmotic significance (Fulda et al., 1999). However, in completely salt-acclimated wild-type cells, proline is not accumulated at significant osmotic levels.

Recently, the accumulation of oligosaccharides (of three to 10 carbohydrate subunits) was shown for *Anabaena* and *Nostoc* strains. These oligosaccharides seem to act as compatible solutes in salt-stressed (Salerno et al., 2004) or heat-stressed (Fischer et al., 2006) cyanobacterial cells. Moreover, the accumulation of mycosporin-like amino acids (MAAs) was reported for not only UV-treated but also salt-treated cyanobacteria (Oren, 1997). However, the MAA amounts found after salt stress were rather low, making its osmotic significance questionable (Portwich & García-Pichel, 1999).

Another possible compatible solute, GGA, was only recently discovered among cyanobacteria. GGA resembles GG, but it is negatively charged (Fig. 2). As might be expected, given their structural similarities, GGA biosynthesis is similar to that of GG; it involves a glucosyltransferase, called glucosyl-phosphoglycerate synthase (GpgS), that uses ADP- as well as UDP-glucose and glycerate-3-phosphate, and the intermediate GGA-phosphate is hydrolyzed to GGA by the specific phosphatase glucosyl-phosphoglycerate phosphatase (GpgP). Using GpgS and GpgP sequences from heterotrophic bacteria, genes encoding these proteins were identified in the genomes of *Synechococcus* 7002 and *Prochlorococcus* SS120. Searches for related proteins in the database indicated that the capacity to synthesize GGA seems to be characteristic of oceanic cyanobacteria not able to fix atmospheric nitrogen (Klähn et al., 2010). However, the role of GGA as a true compatible solute in heterotrophic bacteria is a matter of discussion (Empadinhas & da Costa, 2008b). A similar situation seems to exist for cyanobacteria. GGA is found in only minor amounts and does not play any significant osmotic role among marine *Synechococcus* strains. In these strains, it mainly seems to replace glutamate as the counterion for K\(^+\), as the amount of GGA was regulated by the amount of available nitrogen rather than by the salinity (Klähn et al., 2010). However, in *Prochlorococcus* strains, much higher GGA levels were detected, amounting to up to 50% of the total compatible solute pool, which is dominated by sucrose. Such high amounts are certainly important for osmoregulation and should be compatible with cellular metabolism. Therefore, GGA can be regarded as a fifth compatible solute among cyanobacteria, at least for the genus *Prochlorococcus* (Klähn et al., 2010).

**Transport of compatible solutes**

Transport of compatible solutes is widespread among bacteria. Particularly, heterotrophic bacteria seem to prefer the uptake of these stress protectants over their *de novo* biosynthesis. It is well-documented that model bacteria, such as *E. coli* and *B. subtilis*, can accumulate many more types of compatible solutes than they can synthesize *de novo*. Many uptake systems are well characterized, including high-affinity uptake systems that belong to the group of primary active ABC-type transporters and symporters using H\(^+\) or Na\(^+\) gradients at the cytoplasmic membrane. Their expression and transport activity are stimulated by salt (for review see Bremer & Krämer, 2000). In contrast, cyanobacteria, as photoautotrophic organisms, seem to prefer *de novo* synthesis of compatible compounds, as described above, but employ transporters for the reuptake of their own compatible solutes that have diffused into the periplasm.

The first cyanobacterial transporter for compatible solutes, a multisugar uptake system with specificities for GG, sucrose, and trehalose, has been found in *Synechocystis* 6803 (Fig. 1). Both its gene expression and its transport activity are stimulated by salt, but the former is more pronounced than the latter. In experiments using a mutant unable to synthesize GG, externally added GG was sufficient for the accumulation of high internal GG concentrations and successful salt acclimation (Mikkat et al., 1996). Moreover, in these experiments, GG accumulation preceded the restoration of photosynthesis, cell division, and growth in the GG synthesis mutant, providing evidence for the crucial role of compatible solute accumulation in restoring cellular metabolism (Mikkat et al., 1996; Ferjani et al., 2003). The
PCC 7002
Synechococcus
7120
PCC
Anabaena
PCC 6301
Synechococcus
6803
PCC
Synechocystis
formed with [U-14C]sucrose at a concentration of 1 mM instead of 0.2 mM. Uptake was measured using [14C]GG and [U-14C]sucrose at a concentration
filters and cell surfaces or from impurities of the14C-labeled substrates. Activity during the incubation and resulted probably from binding to
Synechocystis 6803 but is taken up by the transporter, incubation with trehalose, which is not synthesized by

Table 2. Uptake of the compatible solutes (CS) GG and sucrose by different cyanobacteria displaying different salinity tolerance levels and main CS

<table>
<thead>
<tr>
<th>Strains</th>
<th>CS</th>
<th>NaCl (mM)</th>
<th>GG uptake</th>
<th>Sucrose uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synechococcus</td>
<td>GG</td>
<td>2</td>
<td>(19)*</td>
<td>(9)</td>
</tr>
<tr>
<td>PCC 7002</td>
<td></td>
<td>684</td>
<td>953</td>
<td>614</td>
</tr>
<tr>
<td>Synechocystis PCC 6803</td>
<td>GG</td>
<td>2</td>
<td>201</td>
<td>173</td>
</tr>
<tr>
<td>Anabaena PCC 7120</td>
<td>Sucrose</td>
<td>2</td>
<td>211</td>
<td>261</td>
</tr>
<tr>
<td>Synechococcus</td>
<td>Sucrose</td>
<td>342</td>
<td>167</td>
<td>239</td>
</tr>
<tr>
<td>PCC 6301</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anabaena PCC 7120</td>
<td>Sucrose</td>
<td>171</td>
<td>(15)</td>
<td>503*</td>
</tr>
<tr>
<td>Synechococcus</td>
<td>GB</td>
<td>462</td>
<td>(8)</td>
<td>(11)</td>
</tr>
<tr>
<td>PCC 7418</td>
<td></td>
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*Values in parentheses were not derived from linear increase in radioactivity during the incubation and resulted probably from binding to filters and cell surfaces or from impurities of the 14C-labeled substrates.

Fig. 4. Effect of exogenously supplied sucrose, trehalose, and GG on accumulation of compatible solutes in salt-shocked cells of Synechococcus sp. PCC 6301. Cells were grown in BG11 medium and were shocked by 342 mM NaCl for different times. Intracellular levels of sucrose (green), trehalose (orange) and GG (blue) were estimated by HPLC (Mikkat et al., 1997) in cells grown without supplement (a), with 1 mM sucrose (b), 1 mM trehalose (c), and 1 mM GG (d), respectively (S. Mikkat, U. Effmert & M. Hagemann, unpublished data).

The detection of a transporter showing affinities for GG, trehalose, and sucrose, which are characteristic of slightly and moderately halotolerant cyanobacteria, offered a possibility to compare the salt-protective effect of different compatible solutes in vivo. The correlation of the preferred accumulation of GG in moderately halotolerant cyanobacteria and sucrose and/or trehalose in slightly halotolerant strains could indicate that different solutes mediate different salt-resistance levels. Indeed, striking differences in the degree of membrane stabilization have been demonstrated for the different classes of compatible solutes found in cyanobacteria (Hincha & Hagemann, 2004). Therefore, cells of the freshwater strain Synechococcus 6301 were salt stressed in the presence or absence of sucrose, trehalose, or GG (Fig. 4). As expected, the transporter led to the accumulation of trehalose and GG in addition to the de novo synthesis of sucrose in the supplemented cells. The final total amount of compatible solutes was even higher than in cells not supplemented with compatible solutes. While a slightly higher growth rate at 342 mM NaCl (about 2%) was observed, we could not detect a significant increase in the salt-resistance limit of the trehalose- or GG-accumulating cells. Parallel experiments, in which GG was replaced by trehalose in Synechocystis 6803, also resulted in almost unchanged performance under high salt concentrations (Mikkat et al., 1997). In contrast to these results, which indicated a less important role of the type and amount of compatible solute in final salt tolerance, the generation of GB-accumulating Synechococcus strains by genetic
Cyanobacterial salt acclimation manipulation resulted in at least a slight improvement in salt tolerance (Deshnium et al., 1995; Nomura et al., 1995; Waditee et al., 2005). As the expression of Na⁺/H⁺ antiporters was more efficient at improving the salt resistance of *Synechococcus* 7942 (Waditee et al., 2002; Wutipraditkul et al., 2005), the amount or kind of compatible solute seems to be not the main limiting factor for final salt tolerance of this freshwater strain.

Soon after the biochemical characterization of the GG/sucrose/trehalose transporter, the corresponding genes were identified in *Synechocystis* 6803. Downstream of *ggpP* (*stpA*), the gene encoding the GgpP, a gene for a protein with significant similarities to the ATP-binding subunits of ABC the gene encoding the GgpP, a gene for a protein with binding subunit of the salt-regulated proline/choline/GB vicinity of also clearly showed that GG is taken up by a primary active, avoid carbon losses (Hagemann et al., 1997a). The corresponding mutant did not show changes in salt resistance or GG synthesis, but it completely lost the ability to transport GG/trehalose/sucrose. These findings support the assumption that this protein is involved in GG transport, and, accordingly, the gene was named *ggtA*. Moreover, salt-acclimated cells of the mutant lost GG to the medium. Obviously, the main function of this transporter is related to the uptake of GG leaked into the periplasm (Fig. 1) to avoid carbon losses (Hagemann et al., 1997a). These results also clearly showed that GG is taken up by a primary active, ATP-dependent transporter. Usually, those transporters are composed of three or four subunits; however, no candidate gene for the missing transporter subunits was found in the vicinity of *ggtA*. After publication of the complete genome sequence of *Synechocystis* 6803 (Kaneko et al., 1996), the other subunits were identified by systematic mutagenesis. The *ggtBCD* operon encodes the binding protein, GgtB, and the two transmembrane subunits, GgtC and GgtD (Mikkat & Hagemann, 2000). The specificity and affinity of ABC-type transporters is mainly determined by the binding protein. Experiments with purified, recombinant GgtB protein verified the affinity for GG > sucrose >> trehalose and were in agreement with affinity constants measured in intact cells. Last but not least, a salt-induced activation of gene expression was detected for the *ggtBCD* operon as well as for the *ggtA* gene, explaining the approximately fivefold higher transport activity in salt-acclimated cells.

Using the sequence information for the GgtABCD proteins, very similar proteins could be detected in completely known genomes of freshwater and marine cyanobacteria. Moreover, in the genomes of many picoplanktonic *Synechococcus* strains, putative genes for GG/trehalose/sucrose transporters and the GgtABCD-type form a continuous operon and are linked by genes coding for proteins involved in the GG synthesis. These genomic regions very probably harbor the information for GG *de novo* synthesis and a corresponding transporter to avoid loss of organic carbon (Scanlan et al., 2009).

The molecular basis for GB transport was only recently elucidated in cyanobacteria. Evidence for active GB transport was already provided by Moore et al. (1987). They showed active transport of 14C-labeled GB by *A. halophytica* (*Synechococcus* sp. PCC 7418) and some other halophilic, GB-accumulating strains, while this transport activity was missing in strains not able to synthesize GB *de novo*. Moreover, they showed a direct NaCl dependency of the GB transport activity that was taken as a hint for a Na⁺/GB symport mechanism. Recently, the gene for the GB transporter was identified in *A. halophytica* (Laloknam et al., 2006). The BetT transporter is a characteristic member of the salt-regulated betaine–choline–carnitine transporter (BCCT) family bearing significant sequence similarities to the well characterized BetP transporter from *C. glutamicum* and OpUd from *B. subtilis*. However, in contrast to other members of the BCCT family, BetT from *A. halophytica* was specific for GB only. Another remarkable feature of BetT was its alkaline pH optimum, which corresponds to the pH of the natural environment of *A. halophytica*. Moreover, the proposed action as GB/Na⁺ symporter was verified. Functional overexpression in a GB transport mutant of *E. coli* restored its salt resistance in the presence of GB. Similar results were obtained after the expression of *betT* in the freshwater strain *Synechocystis* 7942 (Laloknam et al., 2006).

Another cyanobacterial transport activity is worth mentioning here. The uptake of dimethylsulfiniopropionate (DMSP) has been investigated in water samples from the Gulf of Mexico (Vila-Costa et al., 2006). DMSP is known as a compatible solute in many eukaryotic algae but plays also a role as a sulfur source. Among the phytoplanktonic species, *Synechococcus* and *Prochlorococcus* sp. were identified as taking up DMSP. It is known that DMSP can be taken up through GB-specific transporters. Such transporters have been detected in the genomes of many picoplanktonic strains, including *Prochlorococcus* sp. MIT9313 (Scanlan et al., 2009).

**Kinetics of cyanobacterial salt acclimation**

The salt acclimation of cyanobacteria is a highly dynamic process that is based on the activation/inactivation of many enzymes and transporters as well as a global reprogramming of the general gene expression pattern. Basically, a successful salt acclimation depends mainly on water and ion homeostasis as well as compatible solute synthesis and transport. Dynamic changes in these processes were particularly well studied in the moderately halotolerant strains *Synechocystis* 6803 and 6147 and the freshwater strains *Synechococcus* 7942 and 6301. From these studies, five different acclimation phases were defined for cyanobacterial cells subjected to a sudden salt shock (Fig. 5).
In phase 1, which occurs during the first milliseconds after salt shock, cells shrink because they lose water and many solutes from the cytoplasm. Using an elegant experimental device, Blumwald et al. (1983b) showed the rapid shrinking of the Synechococcus 6311 cells caused by water efflux immediately after salt addition. Probably, many pores or channels at the cytoplasmic membrane open, and not only water but also small water-soluble solutes are lost during this phase, as was shown for K⁺ (Matsuda et al., 2004). A rapid reduction in cell volume has also been shown experimentally by electron paramagnetic resonance (EPR) after applying osmotic shock with sorbitol to Synechocystis 6803 (Shapiguzov et al., 2005). That study revealed that the osmotically driven water loss is mainly due to the aquaporin AqpZ (Fig. 1), as an aqpZ mutant of Synechocystis 6803 did not show shrinking caused by the loss of water immediately after osmotic shock.

The shrinkage of cells is reversed during phase 2, which lasts for several minutes. During this phase, the low osmotic potential of the cytoplasm is increased via the passive influx of external salts, such as Na⁺ and Cl⁻ (Reed et al., 1985b). Ion influx decreases the water potential and causes reuptake of water. The cell volume is restored, but the high internal salt load inhibits cellular metabolism, including photosynthesis and also transcription and particularly translation (e.g. Hagemann et al., 1994; Marin et al., 2004). Therefore, the very early acclimation reactions of the cell rely mainly on the activation of preformed emergency systems and are independent of gene expression.

The following phase 3 is characterized by the exchange of the toxic Na⁺ for K⁺ (Reed et al., 1985b), which occurs during approximately the next hour when Synechocystis 6714 cells were shocked by 500 mM NaCl. This exchange is mainly caused by the activation of pre-existing ion-transport systems, such as Na⁺/H⁺ antiporters and the Mrp and Ktr systems. The newly established ion composition is more compatible with cellular metabolism, allowing the reactivation of photosynthesis to fulfill the energy demand of further ion homeostasis as well as the activation of compatible solute synthesis and gene expression. As mentioned above, the transient influx of Na⁺ may not only act in an inhibitory manner but also have a regulatory function by activating the K⁺ import system Ktr (Matsuda et al., 2004) and compatible solute enzymes, such as GgpS (Hagemann & Erdmann, 1994). A similar regulatory or activating role can be attributed to the transiently high amount of K⁺, especially for gene expression (Gralla & Vargas, 2005).

While phases 1–3 rely mainly on biophysical processes and the activation of pre-existing enzymes, the following acclimation phase 4 involves a reorganization of the gene expression and activity pattern of the whole cell. The prerequisite for this concerted acclimation process is the simultaneous exchange of inorganic ions by compatible solutes. Depending on the amount of salt, during the next 3–8 h, the ion concentrations return almost to the initial levels, and the compatible solute pool is mainly responsible for maintenance of the osmotic potential and turgor pressure. The increase in the relative amount of compatible solutes, such as GG in the case of Synechocystis 6803, is accompanied by a parallel increase of metabolic activities (photosynthesis) and gene expression (transcription followed by translation) (Hagemann et al., 1994; Marin et al., 2004). At the end of this phase, growth and cell division are resumed.

Finally, in phase 5, a new steady-state characteristic of fully salt-acclimated cells is reached after 24 h or more of salt exposure. Such cells are characterized by low internal inorganic ion concentrations and high levels of compatible...
solute concentrations, according to the demands of the external salt concentrations. Depending on the amount of salt, the rates of growth and photosynthesis are somewhat lower than observed before the onset of stress (e.g. Hagemann et al., 1987, 1996). The correlation between dynamic changes in physiological parameters and global changes in gene expression during salt acclimation was recently investigated for the model Synechocystis 6803, at least the above-defined phases 3–5 could be distinguished (Marin et al., 2004).

Genomics, transcriptomics, and proteomics

During the last decade, many new ‘-omics’ technologies have appeared, which have provided valuable databases for the molecular understanding of many processes, including cyanobacterial salt acclimation. The first complete cyanobacterial genome sequence was obtained for the moderately halotolerant strain Synechocystis 6803 (Kaneko et al., 1996). Since then, this strain has served as the main cyanobacterial model for genetic and molecular analyses. This information also facilitated the identification of many genes and proteins necessary for salt acclimation (Fig. 1). For example, in my research group, the genome sequence was used in the characterization of compatible solute synthesis mutants and to identify transport systems for compatible solutes as well as inorganic ions (e.g. Marin et al., 1998; Mikkat & Hagemann, 2000; Elanskaya et al., 2002; Berry et al., 2003). More recently, many additional cyanobacterial genomes have been sequenced and are available in public databases such as Cyanobase (http://genome.kazusa.or.jp/cyanobase/). Among them, genomes from picoplanktonic marine Synechococcus and Prochlorococcus strains dominate, but genomes from many freshwater [e.g. Synechococcus 7942 and 6301, Nostoc (Anabaena) 7120] and moderately halotolerant (e.g. Synechococcus 7002, M. chthonoplastes) model cyanobacteria are also available. Unfortunately, there is not yet a genome sequence from a hypersaline strain (e.g. A. halophytica). Using the knowledge of basic cyanobacterial salt acclimation physiology obtained with a few model strains such as Synechocystis 6803, the increasing number of genome sequences can now be applied to phylogenetic and evolutionary approaches, i.e. to elucidate whether certain salt acclimation strategies are widespread or restricted to specialized strains of cyanobacteria. Particularly interesting are the genomes of picoplanktonic marine Synechococcus and Prochlorococcus strains, as these represent true marine organisms with reduced genome sizes that are believed to contain mainly genes essential for the basic cyanobacterial physiology. Recently, we searched these genomes for proteins potentially involved in basic salt acclimation processes and that could be used to predict different types of salt acclimation (Scanlan et al., 2009).

Based on genome information, DNA microarrays were developed that allow the investigation of global gene expression changes (transcriptomics) under different environmental stimuli. Corresponding experiments were mainly carried out with Synechocystis 6803 by Murata’s group (recently summarized in Los et al., 2008). Transcriptional responses of Synechocystis 6803 to salt stress, as well as many other stresses, were analyzed in detail. These investigations revealed that 200–300 genes are upregulated and that a similar number are downregulated after the addition of salt (Kanesaki et al., 2002; Marin et al., 2003, 2004). These datasets provide a comprehensive overview of all genes encoding proteins directly and indirectly involved in the cellular response of Synechocystis 6803 to salt stress. The high number of differentially regulated genes underlines that an increase in salt concentration causes multiple problems (e.g. due to cationic or anionic ions, water loss, redox changes, higher molecular crowding, and slower growth) for the cell and affects manifold metabolic reactions in a dynamic fashion. In addition to genes for proteins of known function (e.g. those involved in compatible solute biosynthesis and transport, bioenergetic processes, or protein stabilization) a high number of genes encoding hypothetical proteins, whose function is not yet known, were found to be activated. Interestingly, homologs for some of these proteins were also induced in salt-stressed higher plants (Bohnert et al., 2001). On the one hand, these findings can be taken as an indication that the process of salt acclimation is much less well understood than believed, and on the other hand, that some of these unknown mechanisms have been evolutionarily conserved in higher plants. However, the involvement of such proteins in defined acclimation processes remains to be verified in further experiments. Preliminary studies in my group have been conducted to unravel the function of these hypothetical proteins, but inactivation of many of the corresponding genes (e.g. sll1862 – the gene most strongly upregulated after salt shock) did not affect the salt resistance level of Synechocystis 6803.

Furthermore, the available transcriptomic data revealed a large overlap not only between salt and osmotic stress acclimation (Kanesaki et al., 2002; Mikami & Murata, 2003; Shoumskaya et al., 2005) but also with many other stress treatments. In conclusion, the analysis of these datasets allows the distinction of genes or proteins that are specifically regulated by a certain stress and those that are regulated in response to many stress treatments (an in-depth bioinformatic data mining has not yet been performed). The latter group can be defined as general stress-regulated genes/proteins, which are induced by many growth-limiting treatments (Los et al., 2008). Finally, following the kinetics of transcriptional changes allowed the definition of different groups of salt-regulated genes according to their induction time (Marin et al., 2004). Shortly after salt shock
(15–60 min, corresponding to phase 3 defined above), many genes were upregulated; however, the majority of these genes returned to their initial transcription levels in the next few hours. This group of early and transiently salt-induced genes comprises many genes for hypothetical proteins. As the functions of these gene products are often unknown or not related to known stress acclimation reactions, it is hard to predict the roles they play. Their expression is probably not always related to a physiologically meaningful, salt-specific response. A second group of genes becomes maximally expressed around 2 h after the addition of 4% NaCl (corresponding to phase 4 defined above) to Synechocystis 6803 cells. Later on, members of this gene group show a lower but still increased expression level in long-term salt-acclimated cells. The ggpS gene for GG synthesis belongs to this group. A third group of genes becomes upregulated relatively late but remains mostly increased in fully salt-acclimated cells (corresponding to the phase 5 defined above). In all, about 40 genes remain upregulated in cells of Synechocystis 6803 acclimated for several days to 4% NaCl in comparison with cells grown in low-salt medium (Marin et al., 2004). The latter group and some of the genes from the second group encode proteins to which important physiological functions for the acclimation to salt stress can be attributed. Moreover, this study indicates that the selection of the time point for an array experiment may be of crucial importance. To analyze regulatory events and general stress response, rather short times should be analyzed (Los et al., 2008), while the survey of long-term upregulated genes probably reveals mostly stress-specific genes encoding proteins involved in physiologically important acclimation processes (Marin et al., 2004). Transcriptomic studies were followed up by proteome analyses in salt-treated cells. Mostly Synechocystis 6803 was analyzed, using initially the 2D-gel-based technology for the analysis of the periplasmic (Fulda et al., 2000), plasma membrane (Huang et al., 2006), and soluble protein fractions (Fulda et al., 2006). In these studies, the proteins were analyzed by peptide-mass-fingerprinting using MALDI-TOF MS and the quantification was based on quantitative evaluation of stained protein spots. This technology allowed the detection of about 500 proteins; among them, about 50 salt-induced proteins were identified. However, the gel-based technology has limitations, especially in the coverage of membrane-bound or alkaline proteins. Furthermore, quantification of protein by comparing spot-staining intensities is of low sensitivity and accuracy. Therefore, gel-free systems have been developed and applied to proteomics with cyanobacterial cells. The combination of different methods, including prefractionation of proteins and high-throughput MS/MS sequencing, allowed the detection of at least 800 and up to 1100 proteins from Synechocystis 6803 (Gan et al., 2005; Ishino et al., 2007). However, even in these advanced proteomic surveys, only a maximum of 30% of the total proteins from Synechocystis were found, whereas transcriptomic studies are able to display quantitative data for all genes. When the MS/MS approach was applied to the search for salt-stress proteins combined with $^{13}$N-labeling or iTRAQ for quantification (Pandhal et al., 2008b, 2009a, b), similar numbers of proteins were identified as in the 2D-gel approach. Interestingly, these new technologies have allowed cross-species proteomics. The salt-stress response of the nonsequenced halophilic cyanobacterial isolate, Euhalothece sp. BAA001, could be analyzed using the genome information from Synechocystis 6803 (Pandhal et al., 2008a, b, 2009b).

Generally, the proteomic investigations identified many proteins upregulated in salt-shocked or salt-acclimated cyanobacteria. Among them were general stress proteins, such as protein and RNA chaperones, superoxide dismutase, and thioredoxins, but only a few of the expected proteins specifically involved in salt acclimation, such as GgpS, were found (Fulda et al., 2006). Surprisingly, many enzymes involved in basic carbon metabolism were found to be accumulated in salt-treated cyanobacterial cells, which indicates that photosynthetic carbon fixation is severely affected and the overexpression of key enzymes might be a compensation for this. The relation of these proteins to changes in the primary carbon metabolism was recently used for the first systems biology approach to studying salt acclimation (Pandhal et al., 2009a). Moreover, many hypothetical proteins appeared. In correlation with the microarray data, the hypothetical protein Sll1862 was among the most prominent induced spots. There are also indications of post-transcriptional regulation, as no sign of increased transcription was found for many of the salt-induced proteins (Fulda et al., 2006; Pandhal et al., 2008b).

In summary, the ‘-omics’ approaches provided a large quantity of new data on genes and proteins potentially involved in salt acclimation in Synechocystis 6803. However, the quantity of subsequently required experimental work also increased dramatically. As a consequence, detailed bioinformatic processing is required to establish a functional network based on gene induction, kinetics, and coregulation. So far, the ‘-omics’ data have not revealed new processes or mechanisms essential for salt acclimation, but they have provided many ideas for future experiments and insights into the connection of salt acclimation with the whole of cell metabolism.

**Salt-sensing and signal transduction**

Generally, the sensing of salt stress and its subsequent signal transduction to changed gene expression are not well understood. In the model *E. coli*, two two-component systems composed of the Hiks EnvZ and KdpD and their cognate...
Rres, OmpR and KdpE, have been shown to be responsible for the salt-stress-induced regulation of porin genes and of the high-affinity K⁺ uptake system, Kdp, respectively (Mizuno & Mizushima, 1990; Jung & Altendorf, 2002). However, even in these well characterized systems the primary signal sensed is not clearly defined (Wood, 1999). The salt-specific expression of the Na⁺/H⁺ antiporter gene nhaA is regulated by a transcriptional factor, called NhaR, whose promoter binding is modulated by the Na⁺ content (Carmel et al., 1997).

Stimulated by the work in E. coli, the role of two-component systems in stress signaling has been analyzed in great detail with the model Synechocystis 6803, mainly by Murata’s group (reviewed by Los et al., 2008). For these advanced studies, a collection of mutants defective in almost all of the 47 genes potentially encoding Hiks (Suzuki et al., 2000) as well as for the 42 genes potentially encoding Rres (Patthoonrangsarid et al., 2004; Shoumskaya et al., 2005) was generated. These mutant sets were exposed to different environmental stresses, including salt and osmotic stress, and the global changes in gene expression pattern were analyzed by genome-wide transcriptomics using DNA microarrays. This strategy led to the identification of several Hiks (Marin et al., 2003) and subsequently the associated Rres (Shoumskaya et al., 2005) that somehow involved in sensing salt stress. According to these studies, the Hik/Rre pairs Hik33/Rre31, Hik34/Rre1, Hik2/Rre1, Hik16/Hik41/ Rre17, and Hik10/Rre3 are involved in the upregulation of 38 genes 30 min after the addition of 0.5 M NaCl (summarized by Los et al., 2008). However, many genes, including those essential for salt-acclimation processes, such as GG synthesis (e.g. ggpS), remained salt-induced in all two-component mutants tested.

Again, a big overlap was found between the sensing of salt and osmotic stress responses. Mutations in the same set of Hik/Rre-pairs also abolished or diminished the upregulation of the same gene groups after osmotic stress (Patthoonrangsarid et al., 2004; Shoumskaya et al., 2005). However, there were also some genes (e.g. those regulated by the two-component system Hik33/Rre31) that were differently affected by the two stress treatments. Obviously, the same Hik/Rre pair seems to be able to distinguish different stress signals, which was attributed to the association of additional interacting proteins with these sensor modules (Los et al., 2008). Recently, a SipA protein homolog was shown to interact with Hik33 and was able to influence its specificity (Sakayori et al., 2009). Another possibility to explain the differential regulation of salt- and osmotic-shock-regulated genes by identical Hik/Rre systems is that different transcriptional factors interact at the promoters of certain genes.

Moreover, there is an overlap of salt signaling not only with the sensing of osmotic stress, which was expected to a certain extent because both stresses reduce the water available in the surrounding medium, but also with many other stress treatments. Especially the Hik33/Rre31 and Hik34/Rre1 pairs control groups of genes known to be induced by many stress treatments, such as cold, heat, high light, and oxidative stress (Los et al., 2008). New results indicate that the Hik33/Rre31 pair seems to be more specifically involved in the sensing of oxidative stress, as not only is the set of genes controlled by this two-component system very characteristic of oxidative stress acclimation among cyanobacteria, but the corresponding mutant also showed reduced tolerance toward H₂O₂ treatments, whereas salt and osmotic stress tolerance were not affected (Kanesaki et al., 2007). From these data, one can conclude that the Hik33/Rre31 two-component system probably senses and transduces stress signals related to changed energetization of the thylakoid membrane or to the production of reactive oxygen species. This hypothesis would explain the involvement of this two-component system in other stress treatments, as a certain level of oxidative stress is always induced by nonspecific inhibition of PSII or electron transport processes or by an excess of light harvesting in a stressed cell. For the Hik34/Rre1 two-component system, there is good evidence that this system is specifically involved in the heat-shock response of Synechocystis 6803 as a negative regulator (Suzuki et al., 2005). This two-component system regulates mainly genes for chaperones and proteases known to be typical heat-shock proteins, and a corresponding mutant shows the upregulation of this set of genes, resulting in increased resistance toward lethal heat treatments. Again, this set of genes is also induced to a smaller extent by many stress treatments (Los et al., 2008), but one can assume that protein repair and degradation are necessary in most processes of acclimation to harsh environmental stress, including salt and, particularly, heat shock. Obviously, the identified two-component systems are involved in the activation of certain subsets of stress genes involved in more general stress acclimation. In addition to the studies with Synechocystis 6803, there was one report from Nostoc (Anabaena) 7120 showing the involvement of a response regulator in the expression of a gene induced by salt but also by other stresses (Schwartz et al., 1998).

Gene expression levels are usually specified by particular promoter sequences and their interaction with transcriptional factors (e.g. Rres) and RNA polymerase. Sigma factors provide promoter specificity for RNA polymerase in bacteria, and the selection of specific sigma factors is the basis for the reprogramming of gene expression patterns (e.g. Viollier et al., 2003). Besides the principal sigma factor essential for the expression of most genes during exponential growth, bacteria usually employ additional sigma factors that fulfill specialized tasks for their physiology. For example, the group two sigma factor, RpoS, plays an important role in stress acclimation of E. coli, including the stimulation of
osmotic stress-regulated genes (Hengge-Aronis, 1996), and in the Gram-positive model organism B. subtilis, the group three sigma factor, SigB, is involved in the induction of many salt-stress- and general stress-induced genes (Hecker et al., 2007). Cyanobacteria also harbor multiple sigma factors in one cell that may be implicated in specific tasks (for a review see Osanai et al., 2008). Recent analyses have indicated that alternative sigma factors likely function in the acclimation to multiple stresses. The investigation of specific group two sigma factor mutants, especially in the model Synechocystis 6803, indicated that SigB is somehow involved in the general stress response but might be more specifically related to heat shock; SigC plays a role in stationary phase transition and nitrogen regulation; SigD is also involved in many stress acclimations and might more specifically affect light- or oxidative-stress-regulated genes; in contrast, SigE seems to be mostly responsible for the activation of sugar-catabolizing enzymes in cyanobacterial cells grown in the dark or starved of nitrogen (Osanai et al., 2008). Clear conclusions are difficult to draw because group two sigma factors show overlapping promoter recognition with that of the principal sigma factor and also with each other (Goto-Seki et al., 1999); therefore, a mutation in a single sigma factor can be easily compensated for by others. Moreover, the group two sigma factors seem to form a regulatory network influencing their expression and interactions with putative specialized forms of the core polymerase (Osanai et al., 2008).

Interestingly, the sigma factors SigB and SigD have also been found among the genes upregulated by salt stress (Kanesaki et al., 2002; Marin et al., 2003). Moreover, single and especially double mutants defective in these two sigma factors were characterized by a low salt tolerance (Pollari et al., 2008). However, it is not clear whether the lower salt tolerance is a specific effect or it reflects the overall lower stress tolerance, as these mutants also showed a reduction in heat or high light resistance. Unfortunately, it is not known which of the salt-regulated gene inductions are abolished in these mutants. The SigB controls the stress-induced expression of hspA, which encodes a small heat shock protein that acts as a cochaperone (Imamura et al., 2003) and is also strongly induced upon salt shock and remains upregulated in long-term salt-acclimated cells (Marin et al., 2004). Accordingly, a corresponding mutant of Synechocystis 6803 showed a reduced tolerance to salt stress (Asadulghani et al., 2004) and also to light and oxidative stress (Sakhthivel et al., 2009). We provided some evidence that the group three sigma factor SigF might be more specifically involved in the reprogramming of gene expression after salt stress (Huckauf et al., 2000). A Synechocystis 6803 mutant defective in SigF showed not only reduced salt resistance, but also the majority of salt-stress proteins were not further induced in mutant cells, whereas heat-shock proteins were synthesized as in wild-type cells. Moreover, the salt-specific expression of ggpS was strongly delayed, and GG accumulation was also retarded in cells of the SigF mutant (Marin et al., 2002). However, it is still not clear whether SigF represents the missing regulator for induction of salt-stress-specific genes, nor is it clear, if this assumption is correct, how SigF perceives this kind of stress. The promoter consensus sequence of SigF-dependent genes was recently reported (Asayama & Imamura, 2008), but the ggpS promoter does not contain this DNA element. It should be briefly mentioned that the drought-induced extracellular polysaccharide synthesis also seems to rely on the activity of a group three sigma factor, namely SigJ in Nostoc (Anabaena) 7120 (Yoshimura et al., 2007).

Some alternative mechanisms for salt sensing were discussed during the last few years. A special group of adenyl cyclases carrying specific GAF domains was recently identified as potential candidates for a specific salt-sensing molecule in cyanobacteria (Cann, 2007). These enzymes produce the well known signaling molecule cAMP and are influenced specifically by the amount of Na\(^+\) under in vitro conditions. A mutant of Nostoc (Anabaena) 7120 lacking the corresponding genes indeed showed alterations in Na\(^+\)-dependent growth. It could not grow at very low external Na\(^+\) concentrations; however, it was not restricted in its resistance to high salt concentrations (Cann, 2007). Obviously, this specific Na\(^+\) sensor is related to acclimation to sodium limitations rather than to high salt. The phenotype resembled the NahS2 mutant of Synechocystis 6803, which was also not able to resist low Na\(^+\) or an excess of K\(^+\) (Mikkat et al., 2000b). Other interesting candidates are small, noncoding RNAs that have received much attention as a newly discovered group of regulatory molecules (Georg et al., 2009). It has been reported that the noncoding RNA named Yfr1 might be involved in salt sensing, as the corresponding mutant in Synechococcus 6301 showed a reduced salt resistance (Nakamura et al., 2007). However, this mutant was also affected in the oxidative stress response and the expression of the low-carbon-regulated gene sbtA. Further studies are required to unravel the function of these interesting molecules during acclimation processes.

The difficulty in identifying salt-specific sensors is also related to the unresolved question of which primary signal is measured by such a sensor. In the comprehensive review of osmosensing by bacteria, Wood (1999) discussed several possible primary signals specifically linked to osmotic and/or salt stress, including intrinsic strain of membranes, cosolvent effects, ionic strength, and mechanical effects. Clearly, the identification of a defined sensing molecule would help in analyzing this important question in more detail. There are some indications that membrane fluidity might be somehow involved in the sensing of environmental stresses, including salt and osmotic stress, in cyanobacteria.
Many osmotic-shock-induced genes were not activated or does not show water efflux after osmotic shock treatments, aqpZ. Material cells can be explained by the direct modulation of internal ion composition, and molecular crowding, repress movement or processes connected to it, such as turgor, indication that not the membrane tension but the water meter for salt-acclimated cells, these data do not conclusively support the role of turgor as a critical signal. However, despite the clear experimental hints that turgor is an important parameter for salt-acclimated cells, these data do not conclusively support the role of turgor as a critical signal. However, another study pointed more directly to the view that turgor changes might be a signal for initiating alterations in salt-regulated gene expression. After comparing global gene expression in wild-type cells of Synechocystis 6803 and a aqpZ mutant that is defective in the major aquaporin and does not show water efflux after osmotic shock treatments, many osmotic-shock-induced genes were not activated or were activated to a much smaller extent in this mutant (Shapiguzov et al., 2005). These findings were taken as an indication that not the membrane tension but the water movement or processes connected to it, such as turgor, internal ion composition, and molecular crowding, represent a possible signal to be sensed after osmotic shocks.

Finally, many changes observed in salt-treated cyanobacterial cells can be explained by the direct modulation of protein activities via changed internal ion composition, water availability, or molecular crowding; therefore, no specific salt-dependent sensor system regulation, for example a defined transcriptional factor, is necessary in principle. For example, GG synthesis is not only activated in salt-stressed cells at the transcriptional level, but also by activation of the preformed but inactive GG synthesis enzyme, GgpS. It has been shown that different salts are able to perform this activation, and monovalent ions such as K⁺ are especially effective (Schoor et al., 1999). Moreover, the process is reversible, i.e. the salt-activated GgpS can be inactivated by the removal of salt by dialysis and again activated by its subsequent incubation in salt-containing buffers (Hagemann et al., 2001). The direct salt-dependent modulation of enzyme and transport activities has also been shown for other processes essential for successful salt acclimation, such as H⁺-ATPase activity, GG transport, and Ktr activity (Gabbay-Azaria et al., 1994; Mikkat et al., 1997; Matsuda et al., 2004). The direct ion-dependent regulation is not restricted to the modulation of preformed enzymes or protein complexes; it can also regulate differential gene expression. This regulation has been shown for the expression of salt-regulated genes in E. coli. Using an in vitro transcription system, RNA polymerase showed significantly higher affinity toward promoters of salt-regulated genes when K⁺-glutamate was added to the test system, whereas promoters of housekeeping genes were transcribed at much lower rates (Gralla & Vargas, 2005). Therefore, in salt-shocked cells, the remaining RNA polymerase activity is mainly directed to promoters of salt-specific genes, explaining the observed reprogramming of gene expression without any prior signal sensing and transduction system. Such a situation could also be relevant for salt-shocked cyanobacterial cells, as high amounts of inorganic ions, first Na⁺ and later K⁺, are accumulated immediately after salt addition (Reed et al., 1985b), and these high amounts of ions may act as signals. A similar situation also occurs after nonionic osmotic up-shocks, as the high water efflux (Shapiguzov et al., 2005) results in enhanced ion concentrations, especially K⁺, inside the dehydrated cells. This finding may further explain why the changes in gene expression patterns are very similar after salt and osmotic shocks in Synechocystis 6803 (Kanesaki et al., 2002).

In summary, no mechanism for sensing specific salt signals (such as turgor changes, reduced water content, or changes in the ionic composition) has been identified among cyanobacteria. This situation seems to be true for the involvement any two-component system for this purpose, as the ggpS and other salt-specific genes remained unaffected by any of the Hik mutants (Marin et al., 2003). Moreover, none of the Hik mutants of Synechocystis 6803 displayed any reduction in the salt-resistance level, making their direct involvement in the regulation of essential salt-
acclimation processes also questionable. The changed gene expression after salt stress measured by transcriptomics as well as by proteomics obviously represents an integration of acclimation reactions toward different stress signals and clearly shows that salt stress represents a stress syndrome combining many primary inhibitory processes that are most probably sensed by different signal recognition systems. The presently identified two-component systems, namely the Hik/Rre-pairs Hik33/Rre31, Hik34/Rre1, Hik2/Rre1, Hik16/Hik41/Rre17, and Hik10/Rre3, which regulate certain subsets of salt-induced genes, are rather involved in sensing general stress conditions indirectly caused by the addition of salt, such as denatured proteins, reactive oxygen species, or disturbed membrane energetization. To identify salt-stress-specific sensors, one should search for regulatory factors that affect only the expression of specifically salt-regulated genes, such as that for GG synthesis, ggpS. Finally, it could be the case that no defined transcriptional regulator is necessary at all to induce the specific salt response. As was shown for *E. coli*, changes in the internal K\(^+\) concentration could serve as a general signal to display changes in water and ion concentrations (Gralla & Vargas, 2005).

### Evolution of salt acclimation strategies

All extant cyanobacteria display a certain degree of salt tolerance. This principal capability is in line with the view that life evolved in an ancient oceanic environment. The abiotic conditions of the Proterozoic ocean are still uncertain and a matter for discussion. Regarding salinity, there are two opposing views. On the one hand, it is believed that the salt level of the ancient oceans was rather low and increased later because of Na\(^+\) and Cl\(^-\) leaching from the emerging continents. On the other hand, there are arguments for a higher salinity in the ancient ocean than today (see Knauth, 1998). From the evolutionary tree of cyanobacteria, it seems to be more probable that the early ocean was characterized by a lower salinity and higher temperatures, as thermophilic strains displaying a rather low degree of salt tolerance are found at the base of such a cluster analysis (Fig. 6). However, it is uncertain whether the extant cyanobacteria are representative of the strains existing in the Precambrian environment. It cannot be ruled out that the extant cyanobacteria evolved from strains which survived the ‘snowball events’ in Earth history in hot vents of lower salinity, explaining the position of thermophilic, slightly salt-tolerant strains at the base of the extant cyanobacteria. The high impact of these ‘snowball events’ on, for example, the evolution of inorganic-carbon-concentrating mechanisms among cyanobacteria has been clearly recognized (Raven *et al*., 2008). Finally, it should be taken into consideration that the phylogenetic tree construction was conducted using mostly the 16S rRNA gene sequences from strains with completely known genome sequences found in Cyanobase (http://genome.kazusa.or.jp/cyanobase/). This collection is biased by the rather low number of sequenced genomes and the correspondingly low number of axenic cyanobacterial strains, originating from a rather restricted collection of environments.

Nevertheless, the phylogenetic tree clearly shows that the evolution of cyanobacteria did not follow a certain trend of increasing or decreasing general salt tolerance. Moreover, strains are not clustered according to this feature; in most cases, freshwater, marine, and halophilic strains are mixed in one cluster. There are only two exceptions. All marine picoplanktonic strains of the genera *Synechococcus* and *Prochlorococcus* are found in a common cluster. Their separate position inside the cyanobacterial radiation became clear when the proteins involved in inorganic carbon fixation were analyzed. These comparisons showed their principal separation from all other cyanobacteria, leading to the discrimination of these \(\alpha\)-cyanobacteria harboring \(\alpha\)-carboxysomes and RubisCO form 1A from the \(\beta\)-cyanobacteria harboring \(\beta\)-carboxysomes and RubisCO form 1B (Badger *et al*., 2002). Moreover, it has been reported that the *Prochlorococcus* strains evolved from a *Synechococcus*-like ancestor. During this separation, not only pigmentation and other features changed but also the capacity to synthesize compatible solutes (Scanlan *et al*., 2009). Another cluster of low-salt-tolerant strains is made up of the *N₂*-fixing strains with heterocysts of the genera *Nostoc* and *Anabaena* that accumulate sucrose as their sole compatible solute. All these strains occur in freshwater and sometimes in brackish waters, but they never occur as free-living cells in a true marine system. Some related cyanobacteria are found as *N₂*-fixing symbionts inside diatoms (Foster & Zehr, 2006). Interestingly, other *N₂*-fixing strains, such as *Trichodesmium* and *Crocosphaera*, are true marine organisms; therefore, the absence of heterocystic *N₂*-fixing strains from the marine environment does not seem to be directly related to the process of *N₂* fixation but rather to the process of heterocyst formation or its functional maintenance.

Obviously, the different degree of salt tolerance is the result of multiple gene acquisitions or losses over the long cyanobacterial evolution. As explained above, general salt resistance is based on many cellular processes involving multiple genes. Therefore, it is hard to explain the evolutionary path of a certain strain’s salt tolerance accordingly. As shown in Fig. 6, the distribution of genes or the capability for compatible solute synthesis shows such a mixed pattern, corresponding with the view of frequent gene acquisitions by lateral gene transfer among cyanobacteria and other prokaryotes (e.g., Zhaxybayeva *et al*., 2006) as well as frequent gene losses. For example, the freshwater strains *Microcystis* NIES-843 and *Synechocystis* 6803 as well as the marine *Synechococcus* 7002 and the hypersaline strain *Aphanathece* (*Synechococcus* 7418) are phylogenetically very close.
The genome of the *Microcystis* strain NIES-843 did not harbor any gene potentially involved in the synthesis of any compatible solute, not even *spsA* for sucrose biosynthesis, which is normally present in all cyanobacterial genomes. The complete absence of enzymes for compatible solute accumulation reported for these strains or predicted from the genome sequence. Suc, sucrose; Tre, trehalose. 1Compatible solute biosynthesis predicted from the occurrence of corresponding genes in their genome sequences. *No complete genome sequence available; ??, no gene for the synthesis of any known compatible solute in the genome sequence.

(Fig. 6). The genome of the *Microcystis* strain NIES-843 did not harbor any gene potentially involved in the synthesis of any compatible solute, not even *spsA* for sucrose biosynthesis, which is normally present in all cyanobacterial genomes. The complete absence of enzymes for compatible solute biosyntheses explains perfectly its restriction to freshwaters or brackish waters of very low salinity and is most probably the result of multiple gene losses. Another example is the enzymes for GG biosynthesis that most probably originated inside the cyanobacterial radiation before the α-cyanobacteria separated. GgpS proteins forming a cluster neighboring that of other cyanobacteria are found in all marine picoplanktonic *Synechococcus* strains (see Fig. 3). Interestingly, all *Prochlorococcus* strains have lost the ggpS gene but still harbor the gene for the second step in GG synthesis, ggpP (Klähn et al., 2010). Moreover, the ggpP gene from cyanobacteria seems to be transferred into heterotrophic strains, such as *Thiobacillus* (see Fig. 3), whereas the proteins for GgpPs evolved independently within heterotrophic bacteria (Hagemann et al., 2008; Klähn et al., 2010). On the other hand, genes of the *otsAB* type for trehalose biosynthesis have been acquired from heterotrophic bacteria by the marine strain *C. watsonii* as the only example among cyanobacteria, enabling the strain to delete the genes for GG synthesis (M. Hagemann, unpublished data) that are present in all related cyanobacterial strains of this cluster (Fig. 6).

Beside salinity, the available amount of combined nitrogen, one of the major nutrients for photosynthetic cells, seems to have an impact on the distribution of genes for special compatible solute biosyntheses. The capacity to synthesize GGA as secondary compatible solute is restricted to marine cyanobacteria not able to fix *N₂*, as it seems to replace glutamate as a counterion for K⁺ under N-limiting conditions (Klähn et al., 2010). In contrast, among picoplanktonic cyanobacteria, the ability to synthesize the N-containing compatible solute GB is restricted to strains that...
Conclusions and perspectives

During the last 30 years, a comprehensive set of data has been accumulated that allows an advanced understanding of the physiology and molecular biology of cyanobacterial salt acclimation. On the basis of large screening programs, the strain-specific distribution of compatible solutes and its correlation with the salt-resistance level was defined in the pregenomic era. Also at that time, the basic knowledge on internal ion concentrations, ion fluxes across the cytoplasmic membrane, and changes in bioenergetic processes were obtained mostly by applying NMR, EPR, and radiotracer technologies to selected model strains. Regardless of whether they originate from freshwater or hypersaline habitats, all cyanobacteria use the salt-out strategy, which is based on active extrusion of toxic inorganic ions such as Na⁺ or Cl⁻, and the accumulation of organic compatible solutes. This acclimation strategy resembles that of heterotrophic model bacteria, such as E. coli and B. subtilis. In contrast to heterotrophic bacteria, the photoautotrophic cyanobacteria prefer de novo synthesis over uptake for compatible solutes, which is also related to their natural environment in which dissolved organic solutes are not frequently available. The existing osmolyte transport systems mainly serve for the reuptake of compatible solutes leaked into the periplasm. The energy for salt acclimation comes from photosynthesis. During salt acclimation, characteristic changes occur in photosynthetic electron transport, for example the relation between PSII and PSI as well as linear and cyclic electron transfer, respectively, and in the interaction with respiratory processes (this topic was recently reviewed by Allakhverdiev & Murata, 2008).

The publication of the first cyanobacterial genome, from the moderately halotolerant strain Synechocystis 6803 (Kaneke et al., 1996), opened a new era in salt acclimation research. Using this model, genetic approaches generating defined mutants allowed clear conclusions on the proteins and mechanisms underlying basic processes of salt acclimation. Today, Synechocystis 6803 represents the best-known phototrophic bacterium, particularly concerning the physiological processes, molecular mechanisms, and dynamic resolution of salt acclimation. On this basis, it will be possible to analyze potential salt acclimation mechanisms in the many new genome sequences from cyanobacteria, possibly ecologically or economically more important strains. Such genomic screens will give clearer pictures of the evolution of salt acclimation strategies inside the cyanobacterial phylum, which genes were vertically or laterally acquired and possibly lost during the long-lasting adaptation to environments characterized by different salinities or water availabilities. In this regard, it would be very interesting to have a complete genome sequence from a hypersaline strain, preferably A. halophytica (Synechococcus 7418). The existing and future genome information will also be very useful for future application in the generation of salt- or desiccation-tolerant crop plants.

On the basis of an advanced knowledge of cyanobacterial genomics, other ‘-omics’ technologies, such as transcriptomics, proteomics and metabolomics, have been applied to Synechocystis 6803 and a few other strains to analyze acclimation towards many environmental stresses, including salt. The transcriptomic datasets in particular provide a comprehensive list of genes potentially involved in the overall salt acclimation strategy. These data clearly show that many different stress signals act on a cyanobacterial cell upon exposure to increased NaCl concentrations, leading to a very complex response. Nevertheless, the comparison of gene inductions by salt and other stresses will help to distinguish between general stress and salt-specific gene inductions. In this regard, a more advanced evaluation of the existing transcriptomic and proteomic data by advanced bioinformatic tools seems necessary. At present, mainly lists of genes and proteins are available, ordered according to strength of induction and whether they are also induced by other stresses. These selections have mostly been made manually and not using multivariate statistics or other helpful mathematical models.

There are still many important open questions to be solved in the coming years. One important issue, not exclusive to cyanobacteria, is the lack of knowledge on how salt stress is specifically sensed. As outlined above, the known sensory molecules at present are mostly involved in the recognition of signals common to many other stresses. A careful selection of truly salt-regulated genes and proteins should be used as the basis of new analyses. To identify possible regulatory molecules, a systematic promoter analysis of such gene sets would be a good starting point. On this basis, the search for similarities in promoter structure and potential binding proteins (sigma factors, transcriptional factors) and/or the impact of small, noncoding RNAs could be started. Alternatively, strains carrying reporter genes such
as gfp fused to a strong, salt-specific promoter could be used in a systematic mutation screen to identify genes involved in salt-stress signal recognition and/or transition. Another interesting topic will be the functional investigations of the roles of hypothetical proteins. Transcriptome analyses revealed that many genes for such proteins were among the most highly salt-induced genes (e.g. Kanesaki et al., 2002; Marin et al., 2003, 2004). Among these genes are also genes that seem to be specifically regulated by salt stress; moreover, genes for related proteins were also found to be salt regulated in plants (Bohnert et al., 2001). These findings indicate that such proteins may play an important role in successful acclimation to high salt conditions. Their functional characterization will certainly describe new mechanisms involved in this complex acclimation process.

Finally, at present, cyanobacteria receive much attention for their potential application in bioenergy production (e.g. Angermayr et al., 2009) or in solving environmental problems. An advanced knowledge of salt acclimation will also be very helpful to develop these projects further. It is apparent that water will become one of the most valuable resources in the near future. Therefore, the use of saline waters and halotolerant cyanobacteria will most probably be the predominant system for their mass cultivation. Understanding of the salt acclimation processes of the applied strains is certainly necessary to ensure high product yields and avoid side-product formation. Moreover, salinity also affects many soils, reducing the amount of arable land dramatically. The breeding of salt-tolerant crops is urgently needed (Munns, 2005). Genes necessary to ensure high product yields and avoid side-effects will certainly be the predominant strains is certainly necessary to ensure high product yields and avoid side-effects.

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Occurrence of compatible solutes in cyanobacteria.

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