Salt acclimation of *Nodularia spumigena* CCY9414 — a cyanobacterium adapted to brackish water

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ABSTRACT: The toxic cyanobacterium *Nodularia spumigena* regularly forms large surface blooms in the central Baltic Sea. The Baltic Sea is characterized by a salinity gradient. We analyzed the salt acclimation of the strain *N. spumigena* CCY9414, the only *Nodularia* strain with a known genome sequence. *N. spumigena* CCY9414 showed a rather low salt tolerance range, displaying a growth optimum at 12.5 g NaCl l⁻¹. Sucrose was identified as the major compatible solute. The expression of the sucrose-phosphate synthase gene was salt-stimulated, which indicates that the salt-induced sucrose accumulation could be regulated at the transcriptional level. Potassium ions and glutamate were also accumulated in *Nodularia* cells, especially at high salinities when sucrose levels were rather low. Our results indicate that *N. spumigena* CCY9414 represents a truly brackish-water-adapted cyanobacterial strain.

KEY WORDS: Compatible solute · Glutamate · Potassium · Sucrose

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INTRODUCTION

Cyanobacteria are the only prokaryotes that use oxygenic photosynthesis for CO₂ assimilation. Moreover, many cyanobacteria are also able to perform N₂ fixation. The marine diazotrophic cyanobacteria *Trichodesmium erythreum* (Bergman et al. 2013) and *Crocospira watsonii* (Großkopf & Laroche 2012) as well as symbiotic species such as *Richelia* sp. (Foster et al. 2011) and UCYN-A (Thompson et al. 2012) are important primary producers providing organic carbon but also combined nitrogen sources to the marine food chain (e.g. Zehr 2011). However, many cyanobacteria also synthesize bioactive compounds that are toxic to other organisms. Blooms of toxic cyanobacteria have a dramatic impact on the quality of water for drinking or recreation purposes. It has been predicted that harmful cyanobacterial blooms might expand in the future due to global warming (Paerl & Huisman 2008).

Nodularia spumigena, a toxic filamentous cyanobacterium of the group *Nostocales* with heterocysts

for N₂ fixation, regularly forms huge surface blooms in the central Baltic Sea during summer (Wasmund 1997, Stal & Walsby 2000, Sivonen et al. 2007). Recently, it was shown that blooming *N. spumigena* colonies comprise highly productive microenvironments providing ammonia to the microbial community (Ploug et al. 2011). Baltic Sea *Nodularia* strains also are known to produce a variety of bioactive compounds, including nodularin. This cyclic peptide has damaging effects on the mammalian liver (Koskenniemi et al. 2007), and several toxic incidents have been described for the Baltic Sea (Wasmund 2002). The genus *Nodularia* probably comprises different species with diverse ecological specializations (Moffitt et al. 2001, Lyra et al. 2005). Thus, *Nodularia* species are found in coastal waters of different salinities (e.g. see the comprehensive list of *Nodularia* isolates by Lyra et al. 2005), in estuaries (Huber 1984), in salt-affected inland waters (Dorador et al. 2008), and rarely in full sea water (33 g sea salts l⁻¹; Pushparaj et al. 1994).

The Baltic Sea is characterized by a steep salinity gradient with seawater-like salinities at the South-

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west connection to the North Sea and almost freshwater conditions in the Northern and Eastern edges (Samuelsson 1996). The distribution of *Nodularia spumigena* could depend on the external salinity because blooms occur mostly in the central Baltic Sea characterized by brackish waters around 8 practical salinity units (PSU) (Feistel et al. 2010). To adjust the internal osmotic potential to changing salinities, cyanobacteria are known to accumulate specific classes of compatible solutes (Hagemann 2011). Members of the group *Nostocales*, such as the model strain *Anabaena* sp. PCC 7119, usually show optimal growth under fresh water conditions and can acclimate to brackish water salinities by the accumulation of sucrose, which is catalyzed by sucrose-phosphate synthase (SPS) (Porchia & Salerno 1996).

Compared to freshwater *Anabaena* strains, *Nodularia spumigena* seems to prefer brackish water conditions. To get deeper insight into the basic salt-acclimation strategy of this ecologically important cyanobacterial genus, we aimed to investigate the salt response of *N. spumigena* under defined laboratory conditions. For this purpose, we used the Baltic Sea isolate *N. spumigena* CCY9414 (hereafter *Nodularia* CCY9414), taking advantage of its known genome sequence (GenBank accession no. AAVW 000000000; Voß et al. 2013). Genome searches revealed that *Nodularia* CCY9414 is potentially able to synthesize sucrose as well as trehalose as compatible solutes. Our results suggest that *Nodularia* CCY9414 is a brackish-water-adapted cyanobacterium because it grew optimally at salinities around 10 g NaCl l⁻¹ and accumulated sucrose as the main compatible solute under these conditions.

MATERIALS AND METHODS

Strain and cultivation

Nodularia spumigena CCY9414 was obtained from the Culture Collection Yerseke (CCY) at the Netherlands Institute of Sea Research (NIOZ). This Baltic Sea strain was isolated from surface waters of ~9 PSU east of the island of Bornholm. It was taken into the strain collection in 1994. Since that time, *Nodularia* CCY9414 has been maintained in ASN III medium (Rippka et al. 1979) with a reduced NaCl content of 12.5 g NaCl l⁻¹. For our experiments, the cultivation was performed in sterile cell culture bottles (Roth) with ASN III medium, which contained different amounts of NaCl (our standard medium contained 12.5 g NaCl l⁻¹) to mimic different salinities. To com-

pare nitrate-grown cells with cells grown under N₂-fixing conditions, *Nodularia* CCY9414 was cultivated either in ASN III medium (containing 8.82 mM NaNO₃) or ASN III medium devoid of nitrate. The cultures were daily mixed and left to grow up to an optical density at 750 nm (OD₇₅₀) of 0.2 to 0.3 within 2 wk under a 16 h light:8 h dark cycle (with light of 40 µmol photons m⁻² s⁻¹) and a constant temperature of 20°C without CO₂ enrichment. Fresh cultures were inoculated with 2 ml of cells to 50 ml medium (final OD₇₅₀ of 0.01 to 0.02). After 7 d of cultivation, cells from defined volumes were harvested by filtration at the middle of the light period.

Dry mass, chlorophyll, sucrose, and K⁺ determinations

For the chemical analyses, cells from 10 ml of culture were sampled by filtration through glass fibre filters (Whatman GF/F). To determine the dry mass (DM), the cells were harvested on pre-weighed filters and were dried at 105°C overnight. After cooling in a desiccator, the new masses were estimated. The dry masses of cells were corrected for the salt background by subtracting the dry masses of filters through which the same volume of medium of the corresponding salinity was soaked.

For the combined estimation of chlorophyll and compatible solute contents, the filters were immediately frozen in liquid nitrogen and stored at -80°C. Pigments and low-molecular-mass compounds were extracted from frozen filters using 80% acetone (Roth, HPLC grade) at room temperature for 2 h (Porra et al. 1989). Chlorophyll *a* content was estimated spectrophotometrically according to Arnon (1949). The acetone extracts were subsequently dried by vacuum centrifugation. The residues were dissolved in defined volumes of water that contained 50 µg of sorbitol as an internal standard. Low-molecular mass compounds were quantitatively analyzed by gas chromatography (GC; Hagemann et al. 2008) or high-performance liquid chromatography (HPLC; Hagemann et al. 2005).

The potassium ion content was estimated using flame photometry as described in great detail by Mikkat et al. (2000). Inorganic ions were extracted by boiling the filters with 5 ml of double-distilled water for 10 min. Appropriate dilutions were used for the K⁺ estimation with a flame photometer (JENWAY, type PFP7, France). All cell K⁺ values were corrected by K⁺ values from filters, which were treated with the same amount of pure medium of the corresponding salinity.

Gene expression

The expression of selected genes was analyzed using reverse transcriptase PCR (RT-PCR). Samples were taken after incubation at different salinities for 6 h or 7 d. For RNA extraction, 50 ml of cell material was harvested by filtration through glass fibre filters (Whatman GF/F), immediately frozen in liquid nitrogen, and stored at -80°C . To improve the RNA-isolation efficiency, filters were suspended in Buffer RAP (Macherey-Nagel) and homogenized in an ice-cooled cell mill (MM400, Retsch) at maximum speed 3 times for 30 s. Then, RNA was isolated using the Total RNA Isolation Kit from Plant (Macherey-Nagel) as described by the supplier. For reverse transcription, 1 μg of DNA-free RNA was used to generate cDNA with a RevertAid cDNA synthesis kit (MBI Fermentas) using random hexamers as primers. The cDNA amounts were calibrated according to signals obtained by PCR amplification of the constitutively expressed *rnpB* gene (ribonuclease P, *nsp30860*). The PCR program of the Biometra TPersonal Thermocycler started with an initial DNA denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, and DNA synthesis at 72°C for 1 min, and ended with a single final extension at 72°C for 5 min. PCR products were separated in 1% agarose gels and stained with ethidium bromide. The following primers (purchased from Eurofins MWG Operon) were used for the cDNA amplification: constitutive gene *rnpB* (*rnpB_fw*: AAC TAT GAC TAC CCG CCA GCC, *rnpB_rev*: GGC TTT GCC CAA AGC AAA TCG); *spsA* (sucrose phosphate synthase; *nsp8740*; *spsA_fw*: CCC GCA TTA ACT GAG CCA TTT G, *spsA_rev*: TAA CTC GTC GTT GCG GTA ACT G); *sodB* (Fe-containing superoxide dismutase B, *nsp31650*, *sodB_fw*: GAC TCC TCT AAG GTG GGA ATC, *sodB_rev*: CCC AGA CAT CCA AGG TTA AG). The gene-specific primers with an annealing temperature of 52°C were generated with the program Clone Manager Suite using the nucleotide sequences from the *Nodularia* CCY9414 genome available under GenBank accession number AOFE00000000. All primers amplified gene-specific internal fragments of ~300 bp.

Statistical analysis

All experiments were repeated at least twice. In each experiment, the measurements were done as triplicates. Mean values and standard deviations from a typical experiment are shown. Statistical tests

were performed using the 2-tailed Student's *t*-test (Microsoft Excel 10.0).

RESULTS

Cultivation of cells in artificial sea water with or without nitrate addition containing different salt (NaCl) concentrations revealed that *Nodularia* CCY9414 showed a rather narrow range of salt tolerance. It could only grow in the range of 2.5 to 27 g NaCl l^{-1} . Measurements of growth as increase in DM showed an optimum at 12.5 g NaCl l^{-1} (Fig. 1A). At

Fig. 1. Growth of *Nodularia spumigena* CCY9414 at different salinities (in ASN III medium supplemented with NaCl) during 7 d expressed as (A) increase in dry mass (DM) or (B) increase in chlorophyll a (chl a) content. (C) Appearance of N_2 -fixing *N. spumigena* CCY9414 cells. Mean values with standard deviations for cells grown without nitrate, i.e. N_2 -fixing cells, are shown in white columns, while values of nitrate-supplemented cells are presented in grey columns. Values showing statistically significant differences ($p \leq 0.05$) from the values of cells grown at 12.5 g NaCl l^{-1} are marked by an asterisk (*)

salinities $> 20 \text{ g NaCl l}^{-1}$, cells of *Nodularia* CCY9414 grew only very slowly. Moreover, N_2 -fixing cells showed almost the same salt-dependence of growth as nitrate-grown cells.

A different picture appeared when growth was measured in terms of chlorophyll increase. *Nodularia* CCY9414 cells transferred to lower or higher salt concentrations showed only small increases in chlorophyll content compared to cells grown at our standard salinity of $12.5 \text{ g NaCl l}^{-1}$ (Fig. 1B). Cells transferred to the highest salt level of $27.5 \text{ g NaCl l}^{-1}$ even diminished their chlorophyll content in DM. Addition of nitrate clearly supported the chlorophyll production in comparison to N_2 -fixing cells between 5 and 20 g NaCl l^{-1} . Again, the optimal pigment accumulation was observed under brackish conditions at $12.5 \text{ g NaCl l}^{-1}$ (Fig. 1C).

The salt-dependent accumulation of low-molecular mass compounds was analyzed in cells of *Nodularia* CCY9414 acclimated to different NaCl amounts for 1 wk. GC-analysis was used to detect low-molecular-weight sugars and sugar alcohols. This method revealed only 1 major peak, which was identified as sucrose. Trehalose was never detected in *Nodularia* CCY9414 cells by our method. The sucrose amount increased with external salinities and showed maximal levels between 12.5 and 15 g NaCl l^{-1} (Fig. 2A) in N_2 -fixing cells. At the very high salinity of 30 g NaCl l^{-1} , it sharply decreased, probably because cells exposed to very high salt concentrations started to lyse. Similar sucrose contents were measured in cells of *Nodularia* CCY9414 grown with nitrate (Fig. 2B). With this background, all other experiments were carried out with cells grown at only 4 characteristic salinities of 5, 12.5, 20, and $27.5 \text{ g NaCl l}^{-1}$.

The expression of the gene for sucrose-phosphate synthase (*spsA*) was investigated in cells exposed to different salinities by semi-quantitative RT-PCR analysis (Fig. 3). The *rnpB* gene for the RNA subunit of the ribonucleoprotein RNaseP is known as a constitutive gene that served as a reference of cDNA concentrations. When cells were exposed for 6 h to different salinities, a stress-proportional increase of *spsA* mRNA was detected (Fig. 3). However, after salt acclimation for 7 d, the *spsA* mRNA amount was only slightly higher in cells grown at $12.5 \text{ g NaCl l}^{-1}$ than in cells grown in lower salinities, whereas non-growing cells at very high salinities showed only low *spsA* expression. Expression of the gene *sodB*, which is often used as a marker for general or oxidative stress, did not respond to the salt stress treatment of 6 h (Fig. 3).

Fig. 2. Accumulation of sucrose as a major compatible solute in *Nodularia spumigena* CCY9414 at different salinities (in ASN III medium supplemented with NaCl) by (A) N_2 -fixing cells grown under a wide range of salinities and (B) N_2 -fixing and nitrate-supplemented cells grown side by side at selected salinities. Mean values per dry mass (DM) with standard deviations for cells grown without nitrate, i.e. N_2 -fixing cells, are shown in white columns, while values of nitrate-supplemented cells are presented in grey columns. Values showing statistically significant differences ($p \leq 0.05$) from the values of cells grown at $12.5 \text{ g NaCl l}^{-1}$ are marked by an asterisk (*)

Moreover, the contents of soluble amino acids were estimated by HPLC. As usual, glutamic acid was found as the dominating amino acid; it represents almost 50 % of the total pool of soluble amino acids in *Nodularia* CCY9414. The glutamate amount also showed salt-dependent changes. The highest glutamate levels were observed at the highest salt level of $27.5 \text{ g NaCl l}^{-1}$ in both N_2 -fixing and nitrate-grown cells (Fig. 4A). A slight increase in glutamate was also observed in N_2 -fixing cells at the lowest salinity of 5 g NaCl l^{-1} , whereas the cells cultivated at $12.5 \text{ g NaCl l}^{-1}$ showed the lowest glutamate levels. Finally, we estimated the amount of potassium ions in cells of *Nodularia* CCY9414. The K^+ changes at different salinities were similar to the changes in the glutamate levels. The lowest K^+ levels were detected in cells cultivated in the brackish water conditions, whereas the levels increased to significantly higher amounts in cells exposed to the highest salinity of

Fig. 3. Expression of stress-regulated genes in *Nodularia spumigena* CCY9414 at different salinities (in ASN III medium supplemented with NaCl), considering *rnpB*: ribonuclease P, *spsA*: sucrose phosphate synthase, and *sodB*: Fe-containing superoxide dismutase B. Total RNA was extracted from cells exposed to different salinities for 6 h or 7 d. After reverse transcription, the relative amounts of gene-specific cDNAs were detected by RT-PCR

Fig. 4. Accumulation of (A) glutamate and (B) K⁺ in cells of *Nodularia spumigena* CCY9414 at different salinities (in ASN III medium supplemented with NaCl) after 1 wk. Mean values with standard deviations for cells grown without nitrate, i.e. N₂-fixing cells, are shown in white columns, and those for cells grown with nitrate are presented in grey columns. Values showing statistically significant differences ($p \leq 0.05$) to the values of cells grown at 12.5 g NaCl l⁻¹ are marked by an asterisk (*). DM: dry mass

27.5 g NaCl l⁻¹. Under N₂-fixing conditions, an increased K⁺ level was also measured at 5 g NaCl l⁻¹ (Fig. 4B). Compared to sucrose, the glutamate and K⁺ pools were much lower at brackish salinities around 12.5 g NaCl l⁻¹ but reached higher levels at the highest salt concentration. Generally, the addition of nitrate to the growth medium did not change the spectrum and the quantities of compatible solutes in *Nodularia* CCY9414.

DISCUSSION

Our results support the hypothesis that *Nodularia* CCY9414 is well adapted to its brackish water environment. As was found for related strains of the *Nostocales* (Reddy et al. 1989, Fernandes et al. 1993, Moisander et al. 2002), the growth of *Nodularia* CCY9414 is clearly diminished in media containing more than 20 g NaCl l⁻¹. However, in sharp contrast to the studies with freshwater strains of *Nostocales* that always showed the highest growth rates under NaCl-free conditions, the Baltic Sea isolate *Nodularia* CCY9414 showed optimal growth after addition of ~10 g NaCl l⁻¹ and decreased rates under low-salt conditions. The brackish water adaptation of *Nodularia spumigena* is also reflected in its distribution in the natural environment (Fig. 5). In the Baltic Sea, maximal biomass of *N. spumigena* was observed around 7 PSU, and clearly decreased biomass was found at lower as well as higher salinities. Compared to the data of *Nodularia* abundances in the natural salt gradient of the Baltic Sea, the strain *Nodularia* CCY9414 showed higher salt tolerance than observed for the natural Baltic Sea *Nodularia* population. Despite the general difficulties inherent to a comparison of laboratory and field data, this difference could be due to the isolation of a relatively high-

Fig. 5. *Nodularia spumigena* biomass in the natural salt gradient of the central and western Baltic Sea at 0 to 10 m depth between 1979 and 2005. Data based on the Baltic Monitoring Program of the Helsinki Commission (HELCOM)

salt-tolerant ecotype of *Nodularia* near Bornholm, a part of the Baltic Sea with rather high salinities of 7 to 10 PSU. Moreover, the long-term cultivation of this isolate at 12.5 g NaCl l⁻¹ in the stock center CCY might have selected a more salt-tolerant variant of *Nodularia*.

The salt tolerance range (2 to 25 PSU) of *Nodularia* CCY9414 correlates well with the accumulation of sucrose as the main compatible solute. Sucrose is also the principal compatible solute in other *Nostocales* (e.g. *Anabaena* sp. PCC 7119; Porchia & Salerno 1996) and has been discussed as the main reason restricting these cyanobacteria to freshwater and brackish water conditions (Hagemann 2011). The genome of *Nodularia* CCY9414 also harbors genes for the synthesis of trehalose by the TreYZ pathway (Voß et al. 2013). However, we never detected trehalose in cell extracts of salt-treated *Nodularia* CCY9414. Similarly, the *Anabaena* sp. PCC 7120 genome also harbors genes for sucrose as well as trehalose synthesis enzymes. In salt-stressed cells of *Anabaena* sp. PCC 7120, only sucrose is accumulated, whereas trehalose synthesis becomes activated by desiccation stress (Higo et al. 2006).

As expected for a compatible solute, the intracellular concentration of sucrose increased with increasing salt load. However, at very high salinity that did not permit growth of *Nodularia* CCY9414, the sucrose level decreased again. This effect is probably due to unspecific toxic effects in combination with lysis of cells. The stress-proportional increase of sucrose seems to be at least partly regulated by the expression of sucrose-phosphate synthase SpsA. This enzyme has been shown to be mainly responsible for salt-induced sucrose accumulation in *Anabaena* sp. PCC 7119 (Porchia & Salerno 1996). The *spsA* gene of *Nodularia* CCY9414 showed a stress-proportional increase in mRNA contents, while the constitutive gene *rnpB* remained unchanged. We cannot rule out that sucrose synthesis is also salt-stimulated at the enzyme level, as found for enzymes involved in compatible solute synthesis in *Synechocystis* sp. PCC 6803 (Hagemann & Marin 1999, Novak et al. 2011). The expression of the *sodB* gene, encoding the Fe-containing superoxide dismutase induced upon high light, oxidative, and many other stresses (Los et al. 2008), remained unchanged after incubation of *Nodularia* CCY9414 at different NaCl concentrations. This finding was unexpected because salt-stressed cyanobacterial cells often show to some extent responses to oxidative stress, which is mirrored by increased *sodB* transcript levels (e.g. Marin et al. 2004). As a brackish-water-adapted strain,

Nodularia CCY9414 seems to react quite specifically to the salt addition without signs of a general stress situation as indicated by the unchanged *sodB* expression.

In addition to sucrose, we found relatively high levels of glutamate and K⁺ in cells of *Nodularia* CCY9414. Glutamate is often regarded as a minor compatible solute acting as a counter ion for the charged K⁺. In heterotrophic bacteria such as *E. coli*, it has been shown that K⁺ and glutamate strongly accumulate after salt shock, but these compounds are replaced by the major compatible solute trehalose during long-term acclimation (Dinnbier et al. 1988). In *Nodularia* CCY9414, K⁺ and glutamate accumulation was found in osmotically significant amounts, especially under high salt conditions (27.5 g NaCl l⁻¹) but also at the lowest salt level of 5 g NaCl l⁻¹. At brackish water salt levels (here, 12.5 g NaCl l⁻¹) allowing optimal growth of *Nodularia* CCY9414, the amount of sucrose exceeded the total amount of K⁺ and glutamate. Possibly, the importance of K⁺ and glutamate is highest at salinities that inhibit growth and probably also photosynthesis, thus limiting the biosynthesis of sucrose. K⁺ accumulation also was found in salt-stressed cells of related *Anabaena* strains (Apte & Alahari 1994). Non-diazotrophic oceanic cyanobacteria also accumulate glutamate as a minor compatible solute, but under N-limiting conditions, its amount decreases and it becomes replaced by glucosylglycerate (Klähn et al. 2010). *Nodularia* CCY9414 is able to fix N₂, and the glutamate level remained at similar or even slightly higher levels under diazotrophic growth conditions. Moreover, genes for the alternative glucosylglycerate biosynthesis are absent from its genome, as in other marine N₂-fixers (Klähn et al. 2010).

CONCLUSIONS

Our results indicate that strains of the genus *Nodularia* are optimally adapted to brackish water conditions. This conclusion is supported by the observation of optimal growth under brackish water conditions regardless of whether the increase in DM or chlorophyll *a* was used as an indicator. The finding of sucrose as the major compatible solute explains well the usual absence of *Nodularia* from fully marine conditions. Finally, *Nodularia* cells cultivated at salinities characteristic of brackish water bodies showed no sign of general stress in terms of low levels of K⁺ and glutamate as well as constitutive levels of *sodB* expression.

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