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Synthesis of the compatible solutes glucosylglycerol and trehalose by salt-stressed cells of *Stenotrophomonas* strains

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Abstract

In this study, physiological processes were analysed, which are involved in salt acclimation of two *Stenotrophomonas* species, *Stenotrophomonas maltophilia* strain DSM 50170 and *Stenotrophomonas rhizophila* strain DSM 14405. *S. maltophilia* accumulated trehalose as the only osmolyte, whereas *S. rhizophila* produced additionally to trehalose glucosylglycerol (GG). The different spectrum and amounts of compatible solutes in these two strains led to differences in terms of their salt tolerance. The human-associated *S. maltophilia* was able to grow in media containing up to 3% NaCl (w/v). In contrast, *S. rhizophila* propagated in salinities up to 5% NaCl (w/v). The strain was isolated from the rhizosphere, a microenvironment which is characterised by high and changing salinities. Light microscopic analysis of *S. rhizophila* cells showed a significant increase in cell length of salt-treated cells in comparison to control cells. Cells of *S. rhizophila* exposed to more than 2% NaCl excreted GG into the medium during the transition from exponential to stationary growth phase, while the internal trehalose pool remained constant. This feature offers a high potential for the biotechnological production of GG.

Keywords: *Stenotrophomonas*; Salt tolerance; Glucosylglycerol; Trehalose

1. Introduction

The genus *Stenotrophomonas*, which is phylogenetically placed in the γ-subclass of the Proteobacteria [1], was described with the species *Stenotrophomonas maltophilia* [2], previously called *Pseudomonas maltophilia* [3] and later changed to *Xanthomonas maltophilia* [4]. During the last years, some other species have been described, *Stenotrophomonas africana* [5], which is associated with human infections, *S. nitritireducens* [6], which showed an unusual denitrification reaction, and *S. acidaminiphila* [7] isolated from anaerobic sludge blanket reactor. Our group analysed plant-associated *Stenotrophomonas* isolates and could differentiate a new species from environmental sources, named *S. rhizophila* [8].

Stenotrophomonas isolates are widely distributed among nature. They can be found in different types of habitats, such as waters and soil. An association with Eukaryots, such as findings in the rhizospheres of many different plants or isolates of human-associated origin is well documented (for a review, see Denton and Kerr [9]). While many *Stenotrophomonas* strains are potentially pathogenic to humans, others show promising features for biotechnological applications, e.g., for plant growth

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promotion [10]. Particularly, some plant-associated *Stenotrophomonas* isolates show antifungal activity against plant pathogens of economic importance [11–14] and may therefore play a role in the field of biological plant protection [15]. Other isolates of the genus *Stenotrophomonas* became of special biotechnological interest as they are known for their ability to use uncommon carbon sources, e.g., cocaine [16], keratin [17], the explosive RDX [18], and other toxic chemicals [19] making them useful in the field of bioremediation. Moreover, they produce a lot of bioactive substances, such as antibiotics [20], siderophores [15], and compatible solutes [21].

In the last years, interest in compatible solutes (osmoprotective substances, osmolytes) arose in the field of biotechnology. These substances are synthesised and accumulated in response to salt stress. They mediate the protection of biological structures like proteins and membranes, but even whole cells are protected against a decreased water availability caused by increased salt concentrations of the medium or dryness. Compatible solutes are of low molecular weight, show a high solubility and have no interference with cellular physiology even if they are accumulated in high amounts. These substances include several chemical groups, such as carbohydrates (trehalose, sucrose), polyols (glycerol), heterosids (glucosylglycerol – GG), amino acids and derivatives (proline, glycine betaine, ectoines) [22,23]. The species *S. maltophilia* was found to synthesise trehalose as compatible solute whereas the species *S. rhizophila* was able to synthesise GG additionally. While trehalose is a common compatible solute in many bacteria, plants and yeast, GG has been regarded as typical for cyanobacteria [24]. Although GG related compounds like glucosylglycerate are known for soil and plant-associated bacteria like *Erwinia chrysanthemi* [25], GG was recently also found in a limited number of environmental heterotrophic bacteria such as *Pseudomonas mendocina* [26], *P. anguilliseptica* [21] and *S. rhizophila* [8].

Compatible solutes bear the potential for biotechnological applications, e.g., ectoine is used as an additive of enzyme preparations and as a moisturiser in cosmetics [27]. Concerning the production of compatible solutes biotechnological procedures are a promising option in contrast to difficult polyphasic chemical synthesis processes which mostly use toxic solvents. A biotechnological process to produce the osmolyte ectoine was described by Sauer and Galinski [28]. While for trehalose, glycine betaine and ectoines successful applications were shown, no member of the class of heterosides such as GG has been tested. Beside its role as an osmolyte, recently a certain protection of membranes by GG was proven [29]. Ferjani et al. [30] described that the compatible solute GG prevents an inhibition of cell division under salt stress.

The objective of our study was to analyse synthesis of compatible solutes in the type strains of *S. maltophilia* and *S. rhizophila* which is important to understand the ecological behaviour of strains, and to find out the biotechnological potential of these processes. The analysis of GG accumulation in cells of *S. rhizophila* DSM 14405 indicated that this strain shows promising features to establish on its basis a microbial GG production.

2. Materials and methods

2.1. Strains and culture conditions

The examined strains are the type strains of the species *S. rhizophila* DSM 14405 and *S. maltophilia* DSM 50170. DSM 14405 was isolated from the rhizosphere of oilseed rape [15]; DSM 50170 is of clinical origin and was isolated from a patient suffered an oral carcinoma. [3]. Unless otherwise stated, isolates were routinely grown in nutrient broth 2 (Sifin, Berlin, Germany) and stored in broth containing 15% glycerol at −70 °C.

2.2. Physiological characterisation

Salt shock and acclimation experiments were performed in suspension cultures using shaken baffled flasks (100–2000 ml) at 180 rpm and 30 °C. The cells were cultivated in medium according to Palleroni and Doudoroff [31] containing malic acid as carbon source supplemented with different amounts of salt (1–5% NaCl, w/v) and with D,L-methionine (40 mg l^{-1}). In shock experiments, the desired amount of crystalline NaCl was directly added to the cultures at the beginning of the experiments and samples were taken during the exponential and stationary growth phase. For cultivation in a fermenter also medium according to Palleroni and Doudoroff [31] containing 3% NaCl (w/v) was used. The culture volume of 6 liters was introduced to a fermenter of 10 l size (Biostat B, B. Braun, Melsungen, Germany). The fermenter was actuated at 30 °C and 180 rpm. In all cases, the optical density was measured at 600 nm (A_{600}) with a spectrophotometer (Spekol 1100, Carl Zeiss, Jena, Germany). Cells were harvested by centrifugation. Trehalose and GG were extracted from frozen cell pellets by ethanol treatment of lysed cells and incubation at 65 °C for 3 h. Desalting of the samples was carried out by ethanol treatment or using ion exchange resin. The salt-free extract was analysed by HPLC according to Schoor et al. [32]. GG released into the medium was quantified after acid hydrolysis with a blood sugar test kit as described by Erdmann and Schiewer [33]. The content of compatible

solutes was related to the A_{600} or to the total protein content estimated after alkaline hydrolysis of 1 ml cells according to Lowry et al. [34].

2.3. Lightmicroscopic analysis

Stationary phase cells from control and salt-treated (3% NaCl (w/v)) cultures were used for lightmicroscopic analysis. Images of *S. rhizophila* were taken using video-enhanced contrast differential interference contrast (VEC-DIC) as described previously [35] with a Nikon Diaphot 300 inverted microscope (Nikon GmbH, Düsseldorf, Germany) equipped with an oil immersion condenser (numerical aperture 1.4), a 100 × DIC PlanApo oil objective (numerical aperture 1.4) and a mercury arc lamp (HBO 103 W). A C2400-07 Newvicon camera (Hamamatsu Photonics GmbH, Herrsching, Germany) was used in order to acquire DIC images. The analog and digital processing of the DIC images was performed using an ARGUS 20 real time image processor (Hamamatsu Photonics GmbH, Herrsching, Germany). Single-frame images were captured from S-VHS video tapes or directly from the image processor using a LG-3 frame grabber (Scion, Frederick, USA) by the help of IP Lab Spectrum software (Scanalytics, Fairfax, USA). Lengths and widths measurements of *Stenotrophomonas* cells were also carried out using IP Lab Spectrum software after calibration of the measurement area. A population of 130 cells was selected for determining cell size.

2.4. Statistics

In the Tables and figures, means and confidence intervals from three independent experiments are given or the results of one typical experiment are shown. In the figures demonstrating results of lightmicroscopic analysis standard deviations are given and the statistical significance was determined using test according to Mann–Whitney.

3. Results

3.1. Growth at different salt concentrations

In a first series of experiments the salt resistance of two *Stenotrophomonas* species was compared (Table 1). A higher growth rate was found for *S. maltophilia* under low salt conditions compared to *S. rhizophila*. However, salt concentrations exceeding 3%, which conforms to 513 mM NaCl, resulted in an immediate drop in the growth capacity of *S. maltophilia* cells, which showed only very slow growth at 4% and were unable to grow in media containing 5% NaCl. For *S. rhizophila* a different behaviour at increasing NaCl concentrations was found. The growth rate decreased relatively continuously in response to increasing salt concentrations. Even in the presence of 5% NaCl the *S. rhizophila* strain was able to grow albeit with a rather low growth rate.

3.2. Kinetics and amounts of accumulated osmoprotective substances

The total amount of accumulated osmolytes found in cells of *S. rhizophila* increased according to salinity of the medium (Table 1). Whereas the trehalose pool was found to be kept relatively constant, the amount of intracellular GG increased while salinity of the medium ascended. In comparison to the examined *S. maltophilia* strain, the total amount of accumulated compatible solutes was higher in the *S. rhizophila* strain, although the *S. maltophilia* strain showed a faster growth at salinities from 1% up to 3% NaCl (Table 1).

The accumulation kinetics of compatible solutes was compared in cells shocked by different concentrations of NaCl and was followed over the whole growth period. To give an example the accumulation of osmolytes after a salt shock of 1% NaCl is shown (Fig. 1). Immediately after salt shock both *Stenotrophomonas* strains started to accumulate compatible solutes without a significant lag phase. However, while *S. maltophilia* cells accumulated only trehalose during the whole experiment, cells of *S. rhizophila* contained trehalose and GG (Table 1). The accumulation reached a maximal value during the mid

Table 1
Growth rates (μ) and steady state contents of trehalose and glucosylglycerol (GG) in cells of *S. maltophilia* strain DSM 50170 and of *S. rhizophila* strain DSM 14405 grown in the presence of different NaCl concentrations

NaCl (%)	*S. maltophilia* (DSM 50170)		*S. rhizophila* (DSM 14405)		
	μ (h^{-1})	Trehalose (nmol μg^{-1} protein)	μ (h^{-1})	Trehalose (nmol μg^{-1} protein)	GG (nmol μg^{-1} protein)
0	0.42	0	0.36	0	0
1	0.36	5.8	0.26	6.7	2.0
2	0.26	12.5	0.17	12.3	24.2
3	0.18	31.6	0.11	7.9	42.0[a]
4	0.02	28.3	0.07	20.0	64.0[a]
5	0	14.9	0.05	18.5	35.0[a]

[a] Since no real steady state was reached, the values measured after 24 h growth in salt media were taken.

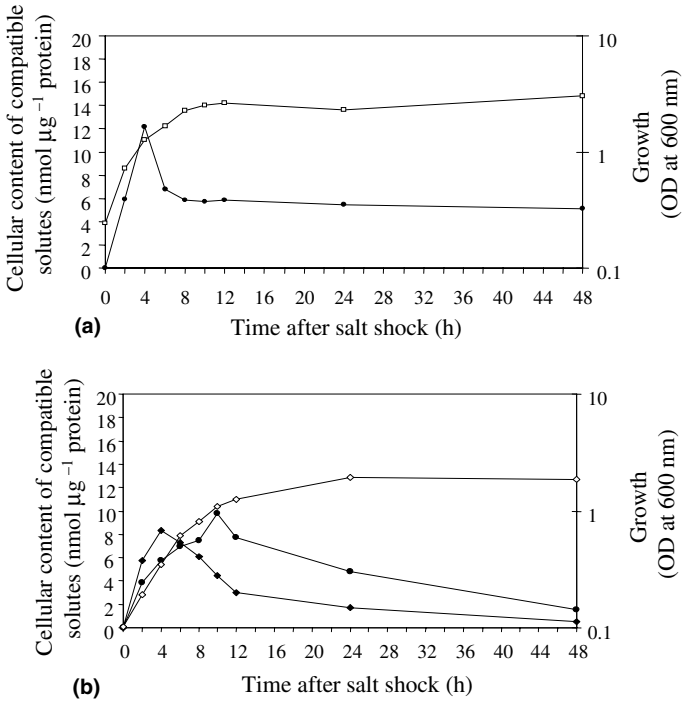

Fig. 1. Accumulation kinetics of compatible solutes trehalose or glucosylglycerol (GG) in cells of *S. maltophilia* DSM 50170 (a) and of *S. rhizophila* DSM 14405 (b) after a salt shock of 1% NaCl. (a) Growth (○) and concentration of intracellular trehalose (●) of *S. maltophilia* DSM 50170 after salt shock in the course of time. (b) Growth (○) and concentrations of trehalose (●) and GG (■) of *S. rhizophila* DSM 14405 after salt shock.

exponential growth phase in salt-shocked cells, while in the late exponential growth phase a slightly decreased steady state value of compatible solutes was observed. This steady state value of trehalose was kept constant in stationary phase cells of *S. maltophilia*. However, cells exposed to more than 3% NaCl showed decreasing cellular amounts of trehalose, which indicates that the dramatically decreased growth rates at high salt concentrations are based on non-sufficient trehalose amounts. In no case trehalose could be detected in the medium. Cells of *S. rhizophila* showed increasing trehalose contents with increasing salt-loading, which was also found for the accumulation of GG up to 4% NaCl and a decreased amount of GG at 5% NaCl. However, while the trehalose levels reached a more or less stable steady state value at the end of exponential growth, the GG values in cells grown in salt concentrations of more than 2% NaCl showed high variability.

Additionally, other carbon sources have been examined in terms of osmolyte synthesis. It was found that in minimal media supplemented with other carbon sources, such as sucrose, glucose, galactose, and xylose, always the two compatible solutes trehalose and GG are accumulated by *S. rhizophila*, while in *S. maltophilia* only trehalose regardless of the C-source was detected.

Surprisingly, cells of *S. rhizophila* shocked by 3% NaCl showed a nearly complete absence of GG in the stationary phase, while the trehalose content remained constant. The decrease of cellular GG during transition of cells into stationary phase is obviously based on a selective release of this compatible solute into the medium, since parallel to the decrease of cellular GG the GG concentration in the medium started to increase (Fig. 2). Nearly the total amount of GG disappeared from the cells and was detected in the medium. Therefore, a controlled degradation of GG can be ruled out. If salt-treated, stationary phase cells from *S. rhizophila* were transferred into fresh, salt-containing medium they showed an immediate activation of growth and GG was accumulated again (data not shown).

3.3. *Cultivation of* S. rhizophila *in a fermenter*

The release of GG into the medium without changing culture conditions could be the basis of a new biotechnological process to produce a compatible solute without cell extraction. To examine the biotechnological usability first experiments in a fermenter have been performed. The maximal cell density measured as A_{600} was approximately 1.5 in the fermenter using the medium according to Palleroni and Doudoroff [31], which contains malic acid as a carbon source and was used before also in the small scale cultivation. In comparison to smaller scale experiments the cell density could not be increased in the fermenter. During the experiment the pH was measured and was found to increase about two units (Fig. 3 (a)). The GG contents within the cells decreased according to growth phase as was shown

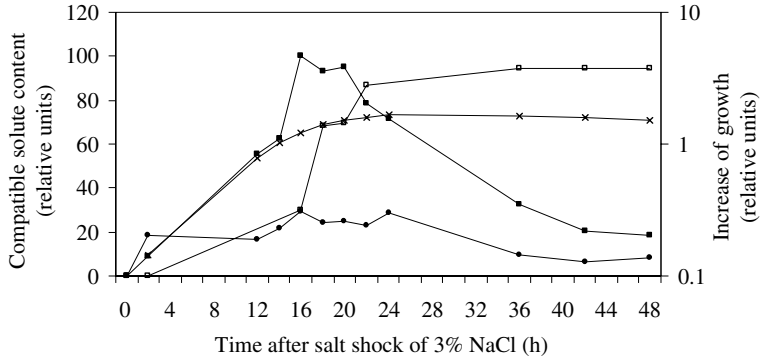

Fig. 2. Export of GG into the medium by *S. rhizophila* DSM 14405 after a salt shock of 3% NaCl in the course of growth. Data of osmolyte concentrations are shown in relative units taking the maximal GG concentration (nmol μg^{-1} protein) as 100% (●: relative intracellular trehalose concentration, ■: relative intracellular GG concentration, □: relative GG concentrations in the supernatant). The growth of cells is indicated by crosses (×).

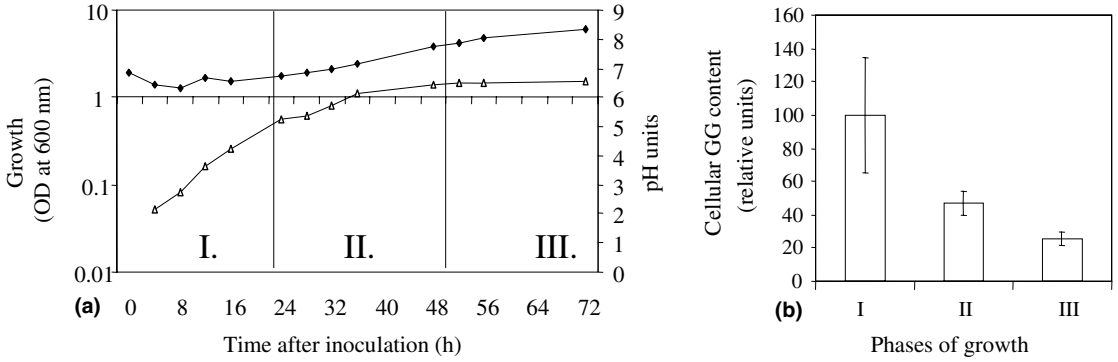

Fig. 3. Cultivation of *S. rhizophila* DSM 14405 in a fermenter. (a) The maximal OD (△) was reached after 40 h and was about 1.5. The pH value (■) was also measured during growth. (b) Intracellular amount of GG during growth.

before. The intracellular amount of GG decreased in 75% (Fig. 3 (b)). After 24 h GG was clearly detectable in the supernatant and was harvested in an amount of 28.7 mg l^{-1} culture broth at 72 h after inoculation.

3.4. Morphology of salt-treated cells

Control and salt-treated cells of *S. rhizophila* were examined by light microscopy (Fig. 4 (a)). It could be demonstrated that salt-treated cells from stationary growth phase show a larger cell size of about 50% in comparison to control cells (Fig. 4 (b)). While the length of the cells was significantly extended, the width of the cells was more or less the same among the examined cell populations, which includes both dividing and non-dividing cells.

3.5. Discussion

Recently, a new species *S. rhizophila*, of the bacterial genus *Stenotrophomonas* has been described [8]. Among the features distinguishing *S. rhizophila* from the *S. maltophilia* differences in the osmolyte spectrum have been found. The complete absence of GG accumulation and the exclusive occurrence of trehalose in any *S. maltophilia* isolate could be verified in this study, in which the osmolyte synthesis was intensively studied under different salt conditions during the whole growth period. The coexistence of GG and trehalose in *S. rhizophila* was also verified leading to much higher total osmolyte contents of salt-treated *S. rhizophila* cells in comparison to *S. maltophilia*. The higher concentration of compatible solutes could be the reason why *S. rhizophila* showed a higher salt tolerance, since it was able to grow at 4–5% NaCl, while *S. maltophilia* only tolerated up to 3% NaCl. A general higher salt resistance could be also shown for GG-accumulating, moderate halotolerant cyanobacterial strains in comparison to low halotolerant strains accumulating only trehalose [36]. On the other side, tolerance to high and changing salinities is important for adaption in the rhizosphere which is the main reservoir for *S. rhizophila* [37]. The ability to synthesise two osmolytes and the resultant higher salt tolerance should be an advantage for such root-associated bacteria. Additionally, *S. rhizophila* like most other heterotrophic bacteria is able to accumulate glycine betaine when cultivated in yeast extract containing media, e.g., Luria Bertani-medium. In the glycine betaine accumulating cells the

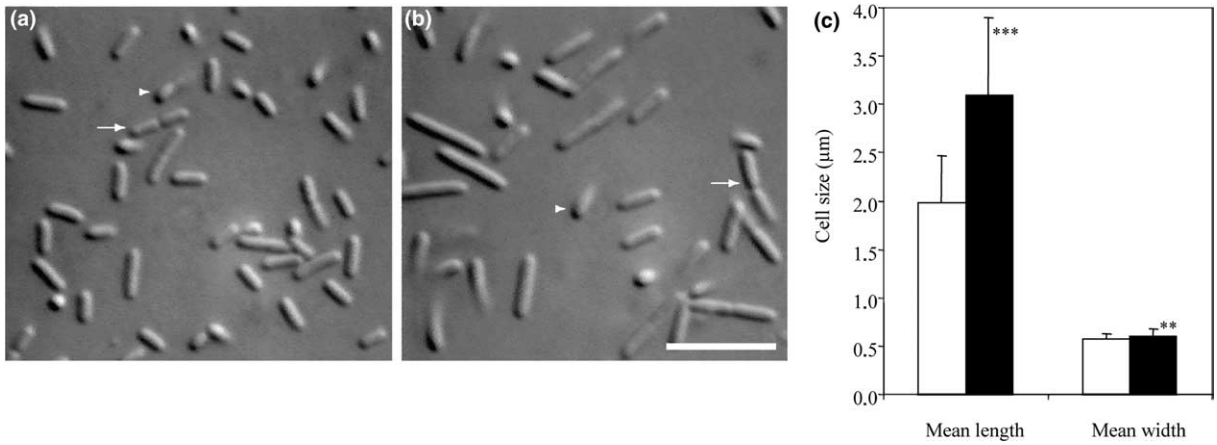

Fig. 4. Lightmicroscopic images using video-enhanced contrast differential interference contrast (VEC-DIC) and morphometric analysis of *S. rhizophila*. Control strain cells (a) are shorter than cells shocked with 3% NaCl. (b) Morphometric measurements of length and width (c) of control cells (white columns) and salt-shocked cells (black columns) confirmed these obvious differences, which were tested for significance using Mann–Whitney test ($n = 130$; $^{**}P < 0.01$; $^{***}P < 0.001$). During morphometric analysis dividing cells (arrows in a and b) were included in the quantification, whereas out-of-focus cells (arrowheads in a and b) were excluded (bar = 5 µm).

GG synthesis is mostly inhibited (unpublished data). The accumulation of glycine betaine at the expense of provided external choline may be also a relevant feature for colonising the rhizosphere.

The parallel occurrence of GG and trehalose in *S. rhizophila* cells grown in minimal medium was restricted to exponentially growing cells. In *S. rhizophila* cells harvested during stationary phase only trehalose could be detected, since GG was nearly completely released into the medium. The immediate release of compatible solutes into the medium is usually only observed, when salt-acclimated bacterial cells were exposed to a down-shock by transfer into low salt medium [38–40]. However, in the case of *S. rhizophila* the release of GG into the medium was related to the transition from exponential to stationary growth phase. It has been shown for many bacteria that the developmental transition to stationary phase results in a general higher stress resistance, which often involves the accumulation of the compatible solute trehalose [41]. Even *in vitro* assays with fragile proteins or macromolecular complexes showed a high protective power of trehalose against diverse stress factors [42]. The remaining trehalose pool seems to be fully sufficient to protect non-growing *S. rhizophila* cells during stationary phase in high salt medium, since after their transfer into fresh salt-containing growth medium they showed an immediate activation of growth and GG accumulation. The GG release from the cells at the transition into stationary phase seems to be highly specific, since no trehalose was leaking out of the cells. This makes it very unlikely that the opening of mechano-sensitive channels is responsible for the GG release, as was found for trehalose release from down-shocked *E. coli* cells [43]. However, a certain critical internal pressure seems to be necessary to induce the selective leakage of GG into the medium, since *S. rhizophila* cells treated by lower salt concentrations of 1–2% NaCl kept the much lower amount of GG stable inside the cell. The physiological meaning of the GG release under the described conditions is difficult to understand in this state of knowledge especially regarding the fact that GG is a worthy compound for the cell synthesised under the effort of organic carbon and energy.

Little is known about the protecting mechanisms of compatible solute in cells. Increasing cell size after exposure to salt loading was described, e.g., for some unicellular cyanobacteria [30,44], *Staphylococcus aureus* [45] and *E. coli* [46]. Concerning *S. aureus* Vijaranakul et al. [45] observed no change in cell size after adding the osmolyte glycine betaine to the culture medium. Ferjani et al. [30] examined the cyanobacterium *Synechocystis* sp. strain PCC 6803 and found that a mutant of this strain, which lacks the gene for GG synthesis, showed increased cell size. They concluded that this effect may be due to a facilitating function of GG in the course of cell division in salt-stressed cells. In this case this effect was reversible by adding GG. In our study, lightmicroscopic analysis of *S. rhizophila* after salt treatment resulted in the observation of increased cell size in comparison to controls. As it was also shown here that the GG content of the cells in stationary phase is rather low, the exceeded cell length may be due to the lack of GG for sustaining cell division under salt stress conditions. These data suggest that the osmolyte GG facilitates not only in cyanobacteria but also in heterotrophic, rod-shaped bacteria like *Stenotrophomonas* cell

division under salt stress. Trehalose, which is also accumulated by *S. rhizophila*, does not seem to play a role for sustaining cell division.

The export of GG in the course of growth by *S. rhizophila* may be a basis for a new biotechnological process for the production of osmolytes. As it was shown by Sauer and Galinski [28] osmolytes like ectoine can be produced in a biotechnological procedure performing an hypoosmotic down shock on *Halomonas elongata* cells. Contrary, a biotechnological process on basis of *S. rhizophila* lacks a costly down shock, since the bacterial cells release GG in the course of growth without changing salinity.

Obtained osmolytes can be used in various industries, for instance as an additive of enzyme preparations in different fields but also as moisturisers in cosmetics for their stabilising properties against biomolecules [27]. For the production of GG there is no biotechnological procedure on whole cell basis described, so far. Takenaka and Uchiyama [47] described and characterised the synthesis of GG by the transglucosidase L-Amano™ (Amano, Chipping Norton, UK) from *Aspergillus niger*. The approach resulted in promising yields but also in the occurrence of by-products. Production systems based on whole cell catalysts excel in a less exceeded extent of demanding procedure parameters such as regeneration of co-factors. Growing *S. rhizophila* in a fermenter did not result in higher cell densities and higher GG yields than had been resulted from former experiments of smaller scale. This fact may be due to the observed increase of pH during cultivation. An optimisation of medium applied should be a basic necessity for the future application of *S. rhizophila* for GG production.

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