Appl Microbiol Biotechnol (2015) 99:5511–5521
DOI 10.1007/s00253-015-6626-x

Identification of mannitol as compatible solute in *Gluconobacter oxydans*

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Received: 17 February 2015 / Revised: 23 March 2015 / Accepted: 25 March 2015 / Published online: 16 May 2015

Abstract *Gluconobacter oxydans* is an industrially important bacterium owing to its regio- and enantio-selective incomplete oxidation of various sugars, alcohols, and polyols. The complete genome sequence is available, but it is still unknown how the organism adapts to highly osmotic sugar-rich environments. Therefore, the mechanisms of osmoprotection in *G. oxydans* were investigated. The accumulation and transport of solutes are hallmarks of osmoadaptation. To identify potential osmoprotectants, *G. oxydans* was grown on a yeast glucose medium in the presence of 100 mM potassium phosphate (pH 7.0) along with various concentrations of sucrose (0–600 mM final concentration), which was not metabolized. Intracellular metabolites were analyzed by HPLC and ^{13}C NMR spectroscopy under stress conditions. Both of these analytical techniques highlighted the accumulation of mannitol as a potent osmoprotectant inside the stressed cells. This intracellular mannitol accumulation correlated with increased extracellular osmolarity of the medium. For further confirmation, the growth behavior of *G. oxydans* was analyzed in the presence of small amounts of mannitol (2.5–10 mM) and 300 mM sucrose. Growth under sucrose-induced osmotic stress conditions was almost identical to control growth when exogenous mannitol was added in low amounts. Thus, mannitol alleviates the osmotic stress of sucrose on cellular growth. Moreover, the positive effect of exogenous mannitol on the rate of glucose consumption and gluconate formation was also monitored. These results may be helpful to optimize the processes of industrial product formation in highly concentrated sugar solutions.

Keywords Acetic acid bacteria · Osmotic stress · Osmoprotection · Glucose dehydrogenase · Membrane protein · Biotransformation

Introduction

Gluconobacter (G.) oxydans is a rod-shaped Gram-negative, aerobic *alpha-proteobacterium* and a member of the family *Acetobacteriaceae* (De Ley et al. 1984). They thrive in their natural sugar-rich environment (like fruit juices, nectars, and honey) by incomplete oxidation of sugars, alcohols, and polyols. The so-called direct oxidative pathways involve the nonphosphorylative oxidation of substrates. The reactions are catalyzed by membrane-bound dehydrogenases that are connected to the respiratory chain. The reactive centers of these enzymes face the periplasmic space. Hence, transport of substrates and products into, and out of, the cell is not necessary. *Gluconobacter* strains are industrially valuable microorganisms due to their nearly quantitative enantio- and regioselective oxidations of hydroxylated carbohydrates (Deppenmeier and Ehrenreich, 2009). Their product spectrum encompasses the vitamin C precursor L-sorbose (Hancock 2009), the precursor of the antidiabetic drug miglitol 6-amino-L-sorbose (Schedel 2000), beverage ingredients (e.g., Bionade from gluconate), and glycerol for the production of the tanning agent dihydroxyacetone (De Muynck et al. 2007). *G. oxydans* is a resilient organism as it survives in highly

Electronic supplementary material The online version of this article (doi:10.1007/s00253-015-6626-x) contains supplementary material, which is available to authorized users.

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acidic environments that have pH values below 3 and also survives high osmolarity, growing in up to 30 % glucose (Sievers and Swings 2005). However, little information is available about its survival mechanisms in such a highly osmotic environment. As in a sugar-rich medium, industrially important bacteria encounter decreased water activity (Aw), which ultimately retards growth and limits biotechnological potential (Kets et al. 1996). Therefore, there is a need to study the physiology and response of *G. oxydans* under osmotic stress. The complete genome of the organism has been sequenced in 2005 (Prust et al. 2005), but osmoprotective mechanisms have not yet been described.

In the present study, we report for first time the role of polyols, especially D-mannitol, as an osmoprotectant in *G. oxydans*. Polyols are well known for their osmoprotective behavior in many organisms, such as mannitol in algae, fungi, yeast (Iwamoto and Shiraiwa, 2005), and some bacteria such as *Pseudomonas putida* (Kets et al. 1996) and *Acinetobacter baylyi* (Sand et al. 2013). We present evidence that *G. oxydans* produces and accumulates mannitol during growth on glucose in the presence of sucrose as an osmoticum. Secondly, mannitol improves the growth of the bacterium when present in low concentrations in highly osmotic media.

Materials and methods

Chemicals and bacterial strain

Media components yeast extract, glucose, mannitol, fructose, sucrose, and cefoxitin were purchased from Carl Roth GmbH (Karlsruhe, Germany). *G. oxydans* 621H Δ*hsdR* (DSM 2343) was obtained from S. Bringer-Meyer, research center Juelich GmbH, Germany.

Growth medium and cultural conditions

G. oxydans 621H Δ*hsdR* was cultured in a yeast extract (0.6 %) mannitol (100 mM) medium (YM) and incubated at 30 °C and 180 rpm. To study the effect of osmotic stress, mannitol was replaced with glucose (50 mM) and 100 mM sterile potassium phosphate buffer (pH 6.8–7.0) was added for maintenance of pH (YGP medium). The osmolarity of the medium was increased by addition of sucrose (0–600 mM). Mannitol was added to the YGP medium at a final concentration of 2.5, 5.0, or 10 mM to study its osmoprotective effect. *G. oxydans* 621H Δ*hsdR* possesses a natural resistance for the antibiotic cefoxitin which was added to a final concentration of 50 μg ml^{-1} in growth media to prevent bacterial contamination. Optical density was measured photometrically at 600 nm (OD_{600}). All cultures were inoculated with 3 % (*v*/*v*) of a preculture grown on the YGP medium. Growth experiments were conducted in at least triplicates using two biological replicates. Representative growth curves are shown. To analyze the effect of mannitol on the rate of glucose consumption and gluconate formation, *G. oxydans* was cultured in a yeast extract (0.6 %) glucose (1000 mM) medium with or without 5 mM mannitol and was incubated under the same conditions as mentioned above.

HPLC analysis

Control and sucrose-stressed cells were harvested at late log phase at 12,000 rpm for 15 min, and the cell pellets were freeze-dried overnight. Intracellular compatible solutes were analyzed by a modified method of Bligh and Dyer (1959; Galinski and Herzog 1990). Briefly, compatible solutes were extracted from 50 to 60 mg of the dried cell material with 500 μl of Bligh and Dyer mixture (10:5:4 methanol: chloroform:water) and vortexed for 5–10 min at room temperature. Then this mixture was extracted using 260 μl 1:1 chloroform:water, vortexed for 5 min at room temperature, and centrifuged at 9300×*g* for 5 min. The upper water and methanol layer was separated and analyzed by HPLC with appropriate dilutions in 80 % (*v*/*v*) acetonitrile. The soluble sugar compounds were separated on a LiChrospher 100 NH_2 column (125×4 mm; 5 μm) (Merck, Darmstadt, Germany) using 80 % (*v*/*v*) acetonitrile and a flow rate of 1 ml min^{-1}. Polyols (e.g., mannitol) and sugars (e.g., fructose, glucose, and sucrose) were identified and quantified by refractive index by comparison to commercial standards. For the analysis of glucose consumption and gluconate formation, 1 ml samples from glucose-stressed cultures were taken at different time points and centrifuged at 10,000 rpm and the supernatants were analyzed on an Aminex-HPX87H column (Bio-Rad, Munich, Germany, 300 mm×7.8 mm) using 5 mM H_2SO_4 as eluent at 65 °C and a flow rate of 0.3 ml min^{-1}. The glucose and gluconate concentrations were determined by refractive index and UV-vis detection, respectively, and quantified by comparison to calibration curves.

^{13}C NMR spectroscopy

For ^{13}C NMR spectroscopy, compatible solutes were extracted from approximately 300 mg of dried cell material essentially as described above. The upper water and methanol layer containing soluble solutes was dried and dissolved in 500 μl of D_2O, supplemented with 5 mg trimethylsilyl propionate sodium salt (TMSP) and 10 μl of acetonitrile (ACN) as internal standards. The NMR spectrum was recorded on a Bruker Avance DPX operating at 75 MHz. Spectra were processed using Bruker TonSpin 1.3 software and calibrated to the residual solvent peaks of TMSP (δ_c=0) and ACN (δ_c=3.61, 121.8). ^{13}C NMR standard shifts: mannitol—*δ*: 73.61, 72.04, 66.05; fructose—*δ*: 104.33, 100.90, 83.50, 78.17, 77.26, 72.51, 72.03, 70.36, 66.70, 66.19, 65.45, 65.22;

gluconate—δ: 181.46, 76.92, 75.41, 74.02, 73.80, 65.46; sucrose—δ: 106.30, 99.77, 84.00, 79.02, 76.58, 75.18, 75.01, 73.68, 71.83, 64.96, 63.97, 62.73; glucose—δ: 97.95, 94.18, 78.01, 77.74, 76.23, 74.76, 73.61, 73.43, 71.74, 62.87, 62.75 (Lewis et al. 2009).

Measurement of protein content

Chloroform was evaporated from the bottom organic phase of Bligh and Dyer extracted samples and hydrated with 10 mM NaOH solution. The protein content was then determined by the Bradford method (Bradford 1976) at 595 nm and quantified using a bovine serum albumin calibration curve.

Microscopy

The morphology of *G. oxydans* cells was observed under osmotic stress and normal growth conditions using a Zeiss Axio inverted microscope combined with a high-resolution fluorescence image system. For phase contrast imaging, 1 μl of the culture was placed on a thin layer of agarose (1 %) in phosphate-buffered saline mounted on a microscope slide and examined with a Zeiss Axio Observer Z1 microscope using a 100x objective (1.46 oil, phase contrast 3). To identify live and dead cells, 20 μl of the culture was mixed with 1 μl of 5 mM SYTOX green dye (Molecular Probes, Life Technologies, Eugene, Oregon) and 1 μl of this mixture was imaged in the same way within 100 ms exposure time using a 38HE filter set at 470 and 524 nm excitation and emission maxima, respectively. An Axiocam MRM camera and the ZEN 2012 software were used for image capture and analysis.

Results

Effect of osmotic stress on growth of *G. oxydans*

Natural habitats of *G. oxydans* are fruits, fruit juices, dates, honey, palm sap, sugar syrups, and drinks like beer (Gupta et al. 2001), which are rich in mono- and disaccharides like glucose, fructose, and sucrose (Chalcoff et al. 2006). Because these habitats display highly osmotic conditions, we were interested in the osmoprotective behavior of *G. oxydans*. Growth was monitored in a YGP medium, and sucrose was used as an osmoticum because of its prevalence in the natural environment of *Gluconobacter* species. Furthermore, *G. oxydans* cannot oxidize sucrose or use it as a carbon and energy source (Fig. 1). Consequently, addition of sucrose does not interfere with glucose metabolism or other metabolic pathways. To increase the osmolarity, the medium was supplemented with 150–600 mM sucrose. In the absence of sucrose-induced osmotic stress, *G. oxydans* had a doubling time of 1.5 h, reaching a final OD_{600} of 1.8 after 25 h

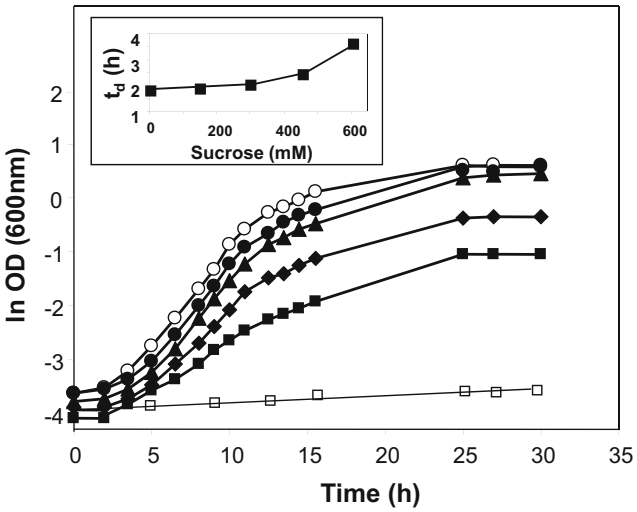

Fig. 1 Effect of increasing sucrose concentrations on the growth of *G. oxydans*. Control without sucrose (*open circles*), 150 mM sucrose (*filled circles*), 300 mM sucrose (*filled triangles*), 450 mM sucrose (*filled diamonds*), and 600 mM sucrose (*filled squares*). The growth medium contained yeast extract-glucose (50 mM) and 100 mM phosphate buffer (pH 6.8). The control culture with yeast extract-sucrose (300 mM) without glucose (*open squares*). The *inset* represents the effect of sucrose concentration on doubling time (t_d)

(Fig. 1). Increasing sucrose concentrations in the medium led to impaired growth as the doubling time increased gradually from 1.5 to 3.6 h. The effect of osmotic stress was most pronounced at sucrose concentrations of 450 and 600 mM where the doubling time increased to 2.5 and 3.6 h, respectively. The final optical density was also severely affected and decreased to 0.7 and 0.5, respectively. The pH of the growth medium decreased from 6.8 to 5.5 regardless of the sucrose concentration (not shown) due to the production of gluconic acid from glucose (Pronk et al. 1989).

Identification of organic osmolytes upon osmotic stress

In many organisms, osmotic stress response is based on the intracellular synthesis and accumulation of compatible solutes. To identify organic osmolytes accumulated in *G. oxydans* in response to osmotic stress, cells were grown in YGP supplemented with 300 mM sucrose to stationary phase. Cells were harvested by centrifugation and freeze-dried, and osmolytes were extracted by a modified method of Bligh and Dyer (1959). To get a comprehensive overview of intracellularly accumulated compounds, the total cellular extract of 300 mM sucrose-stressed cultures was analyzed by ^{13}C NMR spectroscopy (Fig. 2). Under osmotic stress conditions, prominent resonances at 73.42, 71.85, and 65.87 ppm were identified as mannitol by comparison to a commercial standard (Fig. 2). The predominance of these resonances correlated to the presence of a large amount of mannitol observed by HPLC (Fig. S1). Other resonances corresponded to fructose as well as to gluconate and glucose (Fig. 2) which were also identified

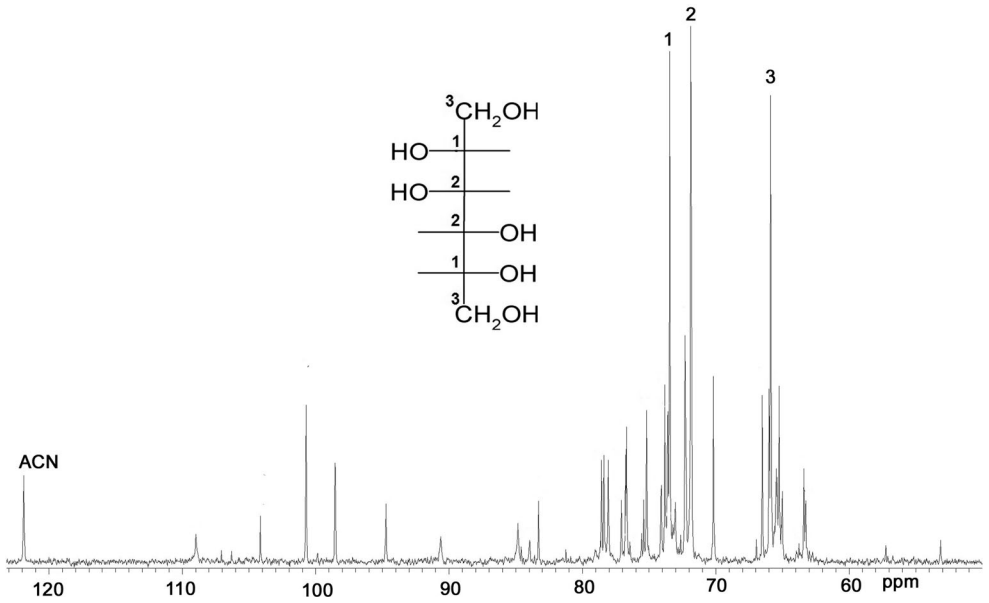

Fig. 2 ^{13}C-NMR spectroscopy of the total cellular extract from *G. oxydans* of a 300-mM sucrose-stressed culture. The spectrum was calibrated to the residual solvent peak of TMSP. Signals observed were from fructose, glucose, gluconate, sucrose, and mannitol. Fructose—δ 104.12, 100.70, 83.27, 78.04, 77.05, 72.28, 71.93, 70.15, 66.49, 65.98, 65.44, and 65.23 ppm. Glucose—δ 98.5, 94.7, 78.54, 78.36, 76.75, 75.37, 74.09, 74.06, 72.29, 63.74, and 63.37 ppm. Gluconate—δ 181.24, 76.75, 75.37, 74.05, 73.74, and 65.44 ppm. Sucrose—δ: 106.30, 99.77, 84.00, 79.02, 76.58, 75.18, 75.01, 73.68, 71.83, 64.96, 63.97, and 62.73 ppm. *Inset*: chemical structure of mannitol and assignment of carbon atoms as shown in this figure

as intracellular components of osmotically stressed cells, but at low amounts compared to mannitol. In total cellular crude extract from the control sample without sucrose addition, only very low resonances were observed for mannitol (not shown). In summary, the data suggest that mannitol was accumulating as an osmoprotectant in stressed cells compared to control cells.

The total cellular extract of cells grown in the presence of 300 mM sucrose was also analyzed by HPLC, and peaks corresponding to glucose, fructose, mannitol, and sucrose were identified by comparison to commercial standards (Fig. S1; Table S1). Mannitol was observed to accumulate in high amounts within the cell despite not being a catabolic product of glucose metabolism by *G. oxydans*. Other compounds observed were sucrose and to a lower extent glucose and fructose. Sucrose was not metabolized and derived from the medium, whereas fructose could be an intermediate of mannitol production. After separation of cells, the supernatant was also analyzed by HPLC and extracellular fructose, and mannitol were not found (not shown). Thus, it was confirmed that mannitol and fructose were synthesized and accumulated intracellularly.

The accumulation pattern of these sugars inside the cells was elucidated by growing *G. oxydans* on YGP media supplemented with 0–600 mM sucrose. The concentration of intracellular mannitol and fructose increased with increasing concentrations of sucrose (Fig. 3). Mannitol and fructose concentrations peaked at 2.8 and 1.4 μmol mg^{-1} protein at the highest sucrose concentration used (600 mM). The intracellular glucose concentration remained almost constant, with a maximum of up to 0.4 μmol mg^{-1} protein at 300 to 600 mM external sucrose concentrations. In the absence of sucrose-induced osmotic stress, fructose accumulation was not observed and only trace amounts of mannitol were seen (0.14 μmol mg^{-1} protein). These results indicated that the intracellular accumulation of mannitol and fructose correlated

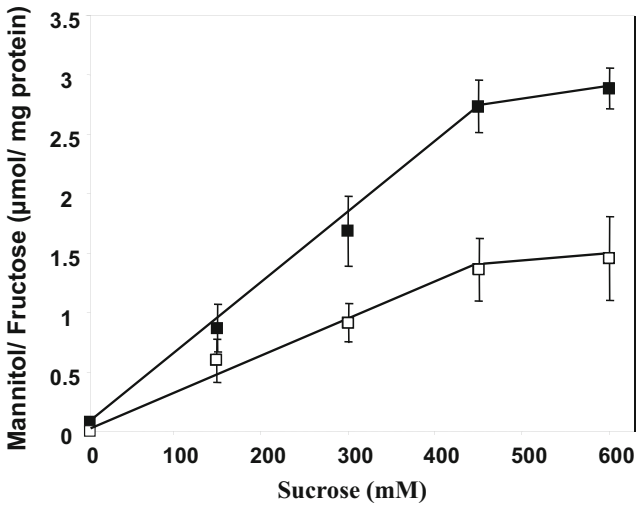

Fig. 3 Effect of extracellular sucrose on intracellular mannitol and fructose accumulation of *G. oxydans*. Cells were cultured in YGP with the addition of sucrose (0–600 mM). Mannitol concentration (*filled squares*), fructose concentration (*open squares*). The figure represents the mean of three independent experiments. *Error bars* represent standard deviation

to extracellular osmolarity of the medium. Consequently, it seems that mannitol plays an important cellular role as a compatible solute in *G. oxydans* under osmotic stress conditions.

Protective effect of extracellular mannitol on *G. oxydans* grown under high osmotic stress

As mannitol is a compatible solute and was produced by *G. oxydans* when exposed to osmotic stress, it was predicted that mannitol should alleviate the osmotic stress from the cells imposed by sucrose. Therefore, the recovery of cellular growth under stress in the presence of extracellular mannitol was investigated. *G. oxydans* was grown in YGP supplemented with either 300 or 600 mM sucrose and 10 mM mannitol (Fig. 4a). Growth of the bacterium under osmotic stress improved significantly in the presence of exogenous mannitol addition. Normal growth behavior was restored when 10 mM mannitol was added to cultures exposed to 300 mM sucrose as the doubling time of cells decreased from 2 to 1.4 h. In contrast, addition of 10 mM mannitol was not sufficient to restore unstressed growth behavior when 600 mM sucrose was used as an osmoticum. However, growth was similar to that of *G. oxydans* exposed to 300 mM sucrose and doubling times decreased from 3.5 to 2 h and cultures reached a final optical density of 1.5 in comparison to 2 obtained by unstressed cultures. The efficiency of low mannitol concentrations on mitigating osmotic stress was then examined by addition of 2.5 or 5 mM to *G. oxydans* exposed to 300 mM sucrose (Fig. 4b). Mannitol concentrations of 2.5 and 5 mM were both sufficient to restore normal growth behavior. Indeed, under these conditions, the doubling times were 1.86 and 1.85 h and the final OD reached 109 and 112 % of the control cultures without osmotic stress. Taken together with the production of mannitol in osmotically stressed cells, these data strongly support an osmoprotectant function of mannitol in *G. oxydans*. Furthermore, a very low amount of extracellular mannitol is sufficient for osmoprotection against 300 mM sucrose.

Effect of mannitol on *G. oxydans* morphology when exposed to osmotic stress

Many acetic acid bacteria form involutions under unfavorable growth conditions, especially *Gluconobacter* sp. (Asai 1971, De Muynck et al. 2007). These involution forms are characterized by enlarged swollen cells, long filamentous cells, or other abnormal morphologies. The presence of these involution forms was investigated in *G. oxydans* exposed to osmotic stress. *G. oxydans* cells grown on YGP were rod or oval shaped (Fig. 5a). Upon addition of 600 mM sucrose, cells formed large swollen irregular globular cells (Fig. 5b) along with some normal cells. Cell shape became less aberrant and was restored to a more normal cell morphology upon addition of 10 mM mannitol to cells exposed to 600 mM sucrose. However, some globular morphologies and swollen cells still persisted (Fig. 5c). To determine if these involution forms were viable, they were stained with SYTOX green (Fig. 5d, e). Upon staining with SYTOX green, all these deformed cells (involutions) were found to be nonviable and comprised almost 20 % of the total cell number in the 600-mM sucrose-stressed culture. On the other hand, 98 % viability was observed when osmotically stressed cells were supplemented with 10 mM mannitol, regardless of their morphology. Compatible solutes protect the cell from osmotic shock by maintaining the intracellular positive turgor pressure, stabilizing the cellular proteins, and maintaining the integrity of biological membranes (Kets 1997). Increased cell viability and mitigation of the involution phenotype in osmotically stressed cells by mannitol addition clearly point to its role as an osmoprotective compatible solute in *G. oxydans*.

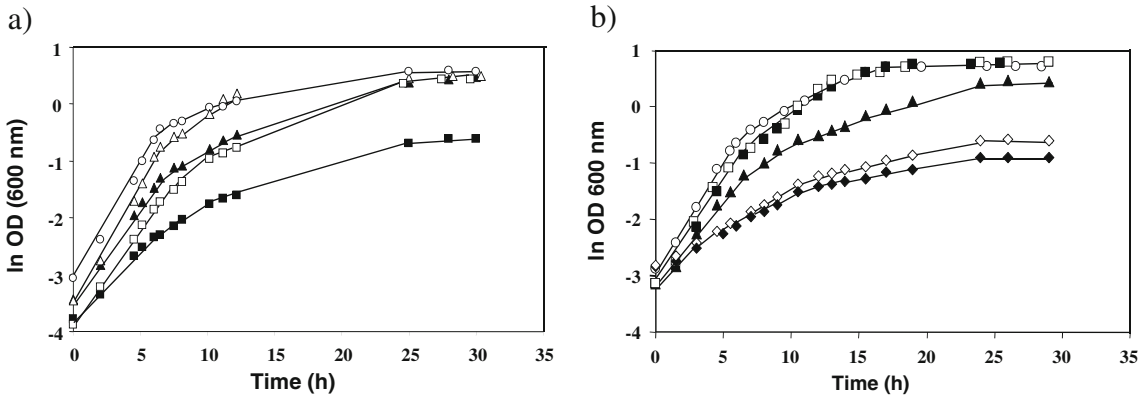

Fig. 4 Effect of extracellular mannitol on the growth of *G. oxydans*. **a** Growth curves in the YGP medium supplemented with 0 mM sucrose (*open circles*), 300 mM sucrose (*filled triangles*), 300 mM sucrose+10 mM mannitol (*open triangles*), 600 mM sucrose (*filled squares*), and 600 mM+10 mM mannitol (*open squares*). **b** Growth curves in the YGP medium with 0 mM sucrose (*open circles*), 300 mM sucrose (*filled triangles*), 300 mM sucrose+2.5 mM mannitol (*filled squares*), 300 mM sucrose+5 mM mannitol (*open squares*), 2.5 mM mannitol (*filled diamonds*), and 5 mM mannitol (*open diamonds*)

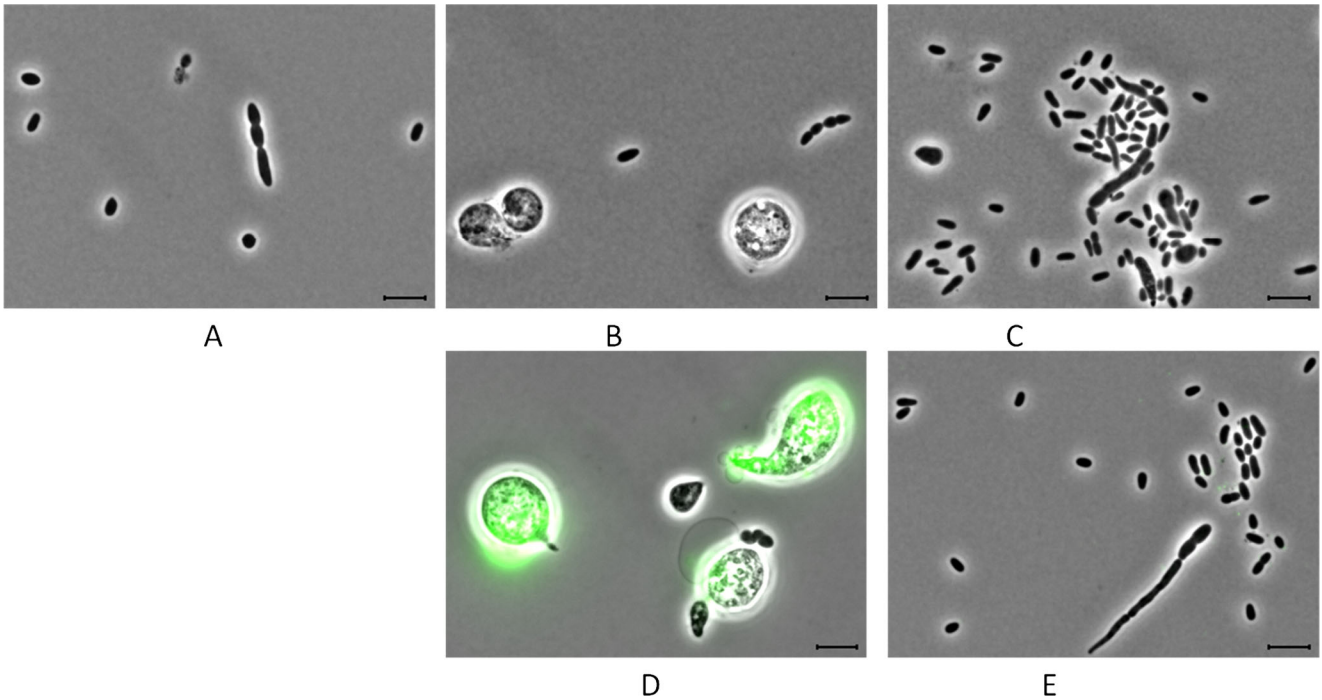

Fig. 5 Microscopic examination of the cells of *G. oxydans*. Phase contract microscopy of *G. oxydans* cells grown in the YGP medium (**a**), YGP medium with 600 mM sucrose (**b**), and YGP medium with 600 mM sucrose+10 mM mannitol (**c**). Fluorescent microscopy of SYTOX green-stained cells with 600 mM sucrose (**d**) and 600 mM sucrose+10 mM mannitol (**e**). *Scale bar*=5 μm

Acceleration of glucose oxidation under osmotic stress

One interesting biotechnological process of *G. oxydans* is the conversion of glucose to gluconate that is used as a sequestering agent and as baking powder. Moreover, gluconate finds application in food and pharmaceutical industries (Meyer et al. 2013). Therefore, the effect of mannitol on glucose oxidation in media containing high concentrations of glucose was investigated. Under these growth conditions, the membrane-bound glucose dehydrogenase catalyzes the oxidation of glucose to δ-gluconolactone in the periplasm, which is then hydrolyzed to gluconate. Only low amounts of glucose are taken up by the cells, which are used for biomass production (Hanke et al. 2013). As expected, the growth of *G. oxydans* in the medium containing 1 M glucose and 100 mM phosphate buffer (pH 7.0) was impaired in comparison to growth in the same medium supplemented with 5 mM mannitol indicated by doubling times of 7 and 1.8 h and a final OD of 0.5 and 2.2, respectively (Fig. 6a). Accordingly, the drop of the pH values during growth of the cultures was different. The addition of 5 mM mannitol led to a decrease of the pH from 7 to 3.5 within 15 h. In the culture without mannitol supplementation, the same decrease was observed after 25 h. The gluconate content of the cultures was monitored to analyze if the addition of the osmoprotectant mannitol correlated to higher gluconate production rates, thus explaining the more rapid drop in pH. Samples were taken at various points during bacterial growth, and glucose and gluconate concentrations were monitored by HPLC. At the end of the exponential growth phase (t=30 h), the osmotically stressed culture produced about 280 mM gluconate in contrast to the culture containing 1 M glucose and 5 mM mannitol that formed 580 mM gluconate within 22 h (Fig. 6b). These results were reflected in the total amount of gluconate production at the end of the experiment (50 h), with about 700 mM gluconate in the mannitol-supplemented culture in comparison to 300 mM gluconate in the stressed culture. Overall, the culture containing 1 M glucose and 5 mM mannitol consumed 750 mM glucose whereas the culture without mannitol degraded only 350 mM of the substrate. The amount of ketogluconates was below 20 mM for both cultures.

Discussion

In the natural environment, microorganisms undergo fluctuations in various physiological parameters like pH, nutrients, temperature, and most importantly water availability. All these variations are based on geological and seasonal effects and have a direct influence on the growth and behavior of microorganisms (Koch 1997). Microorganisms are also quite versatile and adapt to changes in physicochemical parameters such as adjustment of cytoplasmic volume and osmolarity, as well as changing the membrane fatty acid composition under osmotic shifts (Sévin and Sauer 2014; Romantsov et al. 2008)

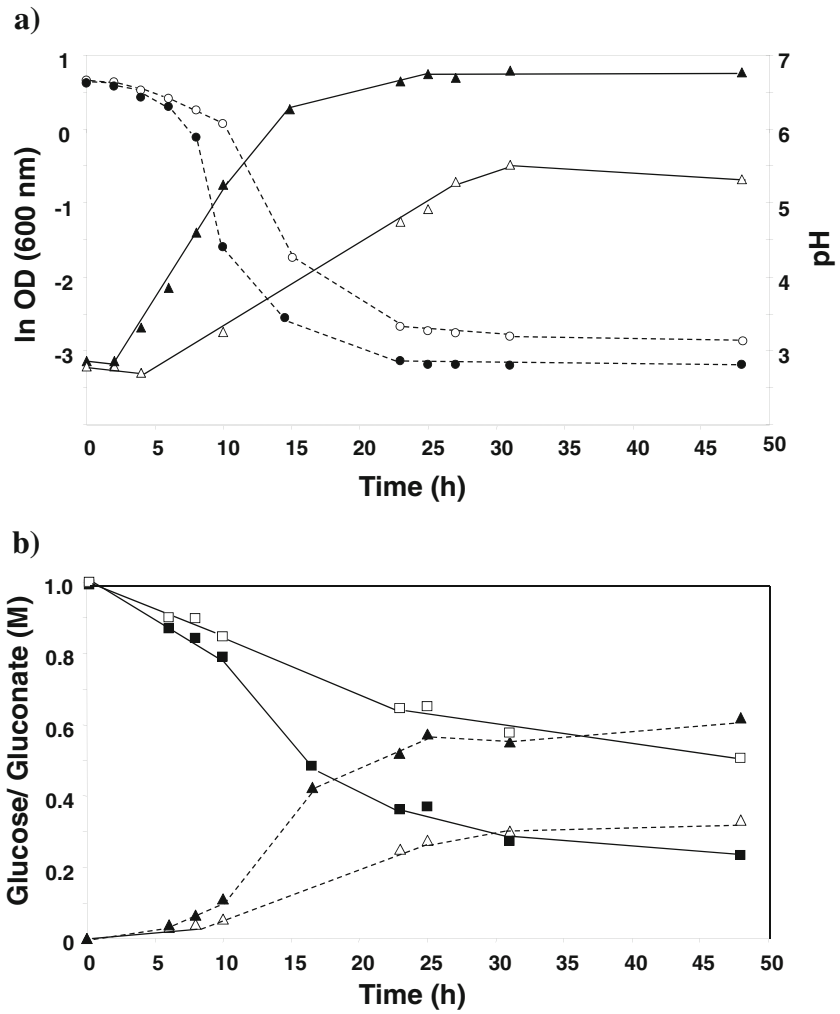

Fig. 6 Effect of mannitol on gluconate production on 1 M glucose-stressed cells. *G. oxydans* was cultured on a yeast extract (0.6 %), glucose (1 M) medium with and without 5 mM mannitol. **a** Correlation between optical density (*open and filled triangles*) and decrease in pH (*open and filled circles)*. **b** Effect of 5 mM mannitol on glucose consumption (*open and filled squares)* and gluconate formation (*open and filled triangles*). *Open symbols* represent the cultures with 1 M glucose, and *filled symbols* represent the cultures with 1 M glucose and 5 mM mannitol. The figure shows a representative experiment from three biological replicates, done in duplicate

and synthesizing compatible solutes under cold and osmotic shock conditions (Sand et al. 2013).

In the present study, we investigated the response of *G. oxydans* to osmotic stress. The organism is able to incompletely oxidize a broad range of alcohols and carbohydrates. This feature is already exploited in several combined biotechnological-chemical procedures for the synthesis of various valuable sugar derivatives (Schedel 2000; Hancock 2009), and for the production of aliphatic and aromatic carboxylic acids that are used as flavoring ingredients (Rabenhorst et al. 2001). However, the productivity and growth of the bacterium decline with increasing sugar concentration of the medium. Due to its industrial importance, this bacterium has been extensively studied but the osmotolerance upon exposure to low water activity has received little attention. However, a recent study showed that *G. oxydans* ATCC 621H and *G. oxydans* DSM 3504 respond quite sensitively to increased osmolality based on the increase of salt concentration by the addition of different buffers. Salt-induced osmolality values of 0.5 osmol kg^{-1} should not be exceeded to avoid inhibition of growth and product formation (Luchterhand et al. 2015). Here, we provided evidence of intracellular accumulation of mannitol as an osmolyte in *G. oxydans* during growth in a medium with high sugar concentrations.

Polyols such as mannitol are known as osmolytes in eukaryotes like fungi, yeasts, and plants. Among bacteria, polyols are rarely synthesized as a response to osmotic stress. However, examples are known in *Zymomonas mobilis*, *P. putida*, and *A. baylyi* (Empadinhas and da Costa 2009). *Z. mobilis* synthesizes and accumulates up to 700 mM sorbitol intracellularly when grown in the presence of 1 M sucrose. This osmoprotectant is produced from sucrose, which is hydrolyzed by sucrase to glucose and fructose. Sorbitol is then formed in the periplasm by a unique glucose-fructose oxidoreductase (EC 1.1.99.-) that oxidizes glucose to gluconolactone and simultaneously reduces fructose to sorbitol. Sorbitol is taken up through energy-dependent sorbitol carriers. Both *P. putida* and *A. baylyi* synthesize mannitol de novo when grown on mineral media containing glucose and succinate as carbon sources, respectively. Gram-positive heterofermentative lactic acid bacteria synthesize mannitol during growth on mixtures of sugars, such as glucose and

fructose (Saha and Racine 2011). Among eukaryotes, different species of yeast and fungi accumulate polyols such as arabitol under xerophytic conditions (Wisselink et al. 2002). Thus, the synthesis and accumulation of polyols as osmoprotectants are found in different groups of organisms and may reflect an evolutionary convergent phenomenon for osmoadaptation.

An osmotic shift affected the growth of *G. oxydans* as the growth rate decreased with increasing extracellular osmolarity of the medium. This decrease in growth rate was gradual (0.4 to 0.19 h^{-1}) and correlated with an increase in external sucrose concentration from 0 to 600 mM. However, the growth of the cells was affected less severely at lower sucrose concentrations, such as 150 and 300 mM, as growth rates were reduced from 0.4 to 0.33 h^{-1} and there was no apparent lag phase observed. On the other hand, at very high osmolarity of the medium (450 and 600 mM external sucrose), the cells were exposed to a severe osmotic shock resulting in low final optical densities and considerably reduced growth rates. Similar effects were found in the closely related bacterium *Z. mobilis* where the growth rate decreased 78 % when sucrose concentration in the medium increased from 200 to 300 g l^{-1} (Sootsuwan et al. 2013). By correlating the effect of increased medium osmolarity on the intracellular osmolyte profile, it was evident that mannitol accumulation increased inside *G. oxydans* with increasing sucrose concentrations in the medium from 0 to 450 mM in a linear fashion. A further increase in the medium osmolarity did not correspond to significantly higher accumulation of the intracellular osmolyte. At 600 mM external sucrose, the internal mannitol and fructose concentrations increased to only 2.8 and 1.4 μmol mg^{-1} protein in comparison to 2.7 and 1.36 μmol mg^{-1} of cellular protein with 450 mM sucrose, respectively. This could be due to the saturation limit of the cell for accumulating these compatible solutes, as well as to the severe growth retardation at higher medium osmolarities. In *P. putida*, the concentration of the compatible solute mannitol increases from 0 to 170 μmol g^{-1} dry weight with increasing NaCl concentration from 0 to 0.5 M. Further increases in NaCl concentration to 0.6 M caused mannitol concentration to decrease to 150 μmol g^{-1} dry weight of the cell. In contrast, the concentration of a second compatible solute Na-acetylglutaminylglutamine amide (NAGGN) increased from 37 to 48 μmol g^{-1} dry weight of the cell with increases of NaCl concentration from 0.5 to 0.6 M in a mineral medium (Kets et al. 1996). ^{13}C-NMR spectra of cellular extracts confirmed the accumulation of mannitol as a major compatible solute in stressed *G. oxydans*. These results are in agreement with findings by Kets et al. (1996) who demonstrated mannitol accumulation in *P. putida* by NMR and mass spectrometry. They additionally found the accumulation of glutamate and NAGGN along with mannitol inside the cell during cultivation on a glucose mineral medium with salts (NaCl, KCl, etc.) and sucrose as osmolytes. In *G. oxydans*, gluconate and fructose were detected along with mannitol rather than glutamate and NAGGN as in *P. putida*. Gluconate is produced by the oxidation of glucose as carbon source, and fructose could be a precursor for mannitol synthesis. The detection of sucrose and glucose is based on the presence of these compounds in the medium that remain in part in the external water space of the cell pellet after centrifugation.

G. oxydans is an obligate aerobic organism and does not grow in the absence of oxygen. In the course of its oxidative metabolism, polyols and sugars are oxidized incompletely by membrane-bound dehydrogenases that channel electrons into the respiratory chain. Products are released into the periplasmic space and are further transported into the medium by porins in the outer membrane. Hence, we can exclude that mannitol is a product from primary metabolism and is not an end product of fermentative processes as found in lactic acid bacteria (Ortiz et al. 2013). Furthermore, mannitol is not a product of glucose consumption in the periplasm or an intermediate of the general catabolism in the cytoplasm. Therefore, it is evident that mannitol is produced as a secondary metabolite in the cytoplasm. In *G. oxydans*, the mechanism of mannitol synthesis under osmotic stress is still unresolved. As mentioned above, small amounts of glucose are taken up by the cells for anabolic reactions. Intracellular glucose is likely oxidized to gluconate by Gox2015, phosphorylated by Gox1709 to 6-phosphogluconate, and channeled into the oxidative pentose phosphate pathway (Rauch et al. 2010). Moreover, Gox1182 was shown to function as glucokinase but displayed only low activity with D-glucose. However, this enzyme could produce sufficient amounts of glucose-6-phosphate as the initial precursor of mannitol. Glucose-6-phosphate isomerase (Gox1704) could then form fructose-6-phosphate (Richhardt et al. 2012), which is then dephosphorylated to yield fructose. Adachi et al. (1999) reported the presence of a NADH and a NADPH-dependent cytoplasmic D-mannitol dehydrogenase in *G. oxydans*. Both dehydrogenases could also catalyze the reverse reaction, namely the reduction of fructose to mannitol. Unfortunately, the amino acid sequences of the proteins are not available for comparison with the proteome of *G. oxydans*. Thus, the genes encoding the D-mannitol dehydrogenases cannot be identified by bioinformatic tools.

The present study also revealed the osmoprotective effect of mannitol on cell physiology and growth when it was provided in small amounts in the medium. It was observed that 2.5–10 mM exogenous mannitol addition could completely mitigate the osmotic stress of growth in 300 mM sucrose as growth rates were similar to those of unstressed cultures. This stimulatory effect of mannitol on the growth was also observed at 600 mM external sucrose as the growth rate of the cells improved from 0.19 to 0.34, but the final optical density was lower in comparison to 300 mM sucrose stress. A similar effect was observed when exogenous compatible solutes were

added to osmotically stressed *Z. mobilis*. In this organism, the final optical density reached 8 when osmotically stressed cells were supplemented with 50 mM sorbitol. Without the addition of sorbitol, the final OD was only 2 (Loos et al. 1994).

Mitigation of osmostress in *G. oxydans* can also be accomplished through import of mannitol. The fact that external mannitol added to low concentrations could restore normal doubling times and growth yields under osmotic stress conditions indicated that there is a transporter for mannitol in *G. oxydans* that facilitates its uptake into the cytoplasm. Mannitol could also be oxidized to fructose in the periplasm by the catalytic activity of the PQQ-dependent sorbitol dehydrogenase (Hancock 2009). In this case, fructose would be taken up and reduced to mannitol in the cytoplasm. Under these conditions, de novo synthesis of mannitol or fructose in the cytoplasm is not necessary. The synthesis of compatible solutes is energy dependent and expensive; therefore, microorganisms prefer the uptake from the environment rather than de novo synthesis (Oren 1999). Hence, the presence of this organic osmolyte explains the positive effect of external mannitol on growth under osmotic stress.

Mannitol uptake has been analyzed in *Escherichia coli* and other bacteria such as *Streptococcus mutans*, *Bacillus stearothermophilus*, *Bacillus subtilis*, *Clostridium acetobutylicum*, and *Lactococcus lactis* (Groisillier et al. 2014). In these bacteria, mannitol is taken up by a mannitol-specific phosphotransferase system (PTS). In *G. oxydans*, the transporter for the uptake of mannitol is unknown. It is unlikely via a similar PTS as homologues to EIICD transporter proteins are not found in the genome of *G. oxydans* (Prust et al. 2005). In contrast to other bacteria, *Pseudomonas fluorescens* and *Phaeobacter inhibens* DSM17395 import mannitol via specific ABC transporters (Wiegmann et al. 2014). A couple of sugar/polyol-dependent ABC transporters were also identified in *G. oxydans* that might be involved in mannitol uptake (e.g., Gox1179-1184, Gox2182-2185, Gox2219-2221). Alternatively, mannitol could be imported by putative sugar/polyol-proton symporters that are encoded on the genome of *G. oxydans* (e.g., *gox1971*, *gox1047*, *gox0925*, *gox080*, *gox0649*).

The osmoprotective behavior of the mannitol could be linked to maintaining the cell turgor pressure, stabilizing the cellular membranes (fatty acids and proteins) and protecting the cell from oxygen radicals generated under low water activity (Jennings 1984; Kets 1997). In another industrially important bacterium, *Lactococcus lactis*, mannitol was shown to be a cryoprotectant and enhanced cell viability under drying conditions (Efiuvwevwere et al. 1999). Thus, it may be that mannitol protects cellular enzymes by excluding water from the macromolecular interface and maintaining native structure and function (Arakawa and Timasheff 1985). From microscopic examination and SYTOX staining of *G. oxydans*, it became clear that mannitol is involved in maintaining the viability of cells under low water activity. The morphology of the cells was more severely affected in the medium with sucrose in the absence of exogenous mannitol, and these deformed cells were less viable. In the presence of exogenous mannitol along with sucrose, the severity of deformity and limited viability was alleviated.

Bacteria are often grown under osmotically stressful conditions in industrial settings, such as use of high osmolyte concentrations or growing cells to high densities. Osmotic stress is linked to mutations, decreased biocatalytic rates, and growth yields (Dragosits et al. 2013; Shiloach and Fass 2005; Winkler et al. 2014). Therefore, mitigating osmotic stress by addition of an osmoprotectant or increasing de novo synthesis of an osmoprotective compatible solute is essential for long-term, industrial-scale fermentation. Growing bacteria to high densities is often required for high volumetric productivity needed to produce recombinant proteins and industrial products. This is especially true for low-value, high-volume products (e.g., succinic acid, acetone, styrene oxides, and PHB) as well as for high-value products (e.g., cosmetics and nutraceuticals) since economic feasibility requires reduced fermentation costs (Lee 1996). Hence, increasing and maintaining high cell densities are critical. However, media ingredients added to high concentrations often become inhibitory, especially osmolytes (Riesenberg et al. 1991). Indeed, decreased glucose consumption and gluconate yields were observed when *G. oxydans* was grown in 1 M glucose (Fig. 6b). Addition of the osmoprotectant mannitol restored normal growth rates and yield, while also doubling gluconate yields. Consequently, the production of high quantities of gluconate in media containing concentrated sugar solutions is aided by the addition of mannitol in *G. oxydans*. Large-scale production of gluconate is biotechnologically desirable since gluconates find application in many industries. For example, foodstuff and pharmaceutical industries use gluconate because it is nontoxic, soluble, and stable even when sterilized. Additionally, gluconates are used in the production of foodstuff as leavening agent, and in disk washing powders for chelating Ca^{2+} ions (Prescott et al. 1953; Sawyer 1964; Mattey 1992). Furthermore, osmotolerance of *G. oxydans* is one of the most important physiological characteristics for the conversion of a highly concentrated sugar/polyol solution for the biotechnological production of value-added products such as L-sorbose (vitamin C production), 6-amino-L-sorbose (miglitol synthesis), and dihydroxyacetone from glycerol. Hence, biocatalyst robustness toward stresses imposed during fermentation processes is important for efficient bio-based production. The identification of mannitol as a compatible solute will expand our current knowledge of the osmotic stress response in *G. oxydans* and will be useful for the rational engineering of osmotic-tolerant strains to improve processes in future applications.

Acknowledgments We thank Dr. Fabian Grein from the Institute of Microbiology and Biotechnology, University of Bonn, for assisting in microscopic examination of cell cultures. Special thanks to the Higher Education Commission of Pakistan and their German collaborators DAAD for financial support (scholarship for NZ). We thank Elisabeth Schwab and Marlene Hecker for technical assistance.

Conflict of interest There is no conflict of interest for all authors.

References

Adachi O, Toyama H, Matsushita K (1999) Crystalline NADP-dependent D-mannitol dehydrogenase from *Gluconobacter suboxydans*. Biosci Biotechnol Biochem 63:402–407

Asai T (1971) The classification and biochemistry of acetic acid bacteria. In: Sakaguchi K, Vemuna T, Kinoshito S (eds) Biochemical and industrial aspects of fermentation. Kadansha Ltd., Tokyo, pp 201–232

Arakawa T, Timasheff SN (1985) The stabilization of proteins by osmolytes. Biophys J 47:411–414

Bligh EG, Dyer WJ (1959) A rapid method of lipid extraction and purification. Can J Biochem Physiol 37:911–917

Bradford MM (1976) Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Chalcoff VR, Aizeni MA, Galetto L (2006) Nectar concentration and composition of 26 species from the temperate forest of South America. Ann Bot 97:413–421

De Ley J, Gillis M, Swings J (1984) The genus *Gluconobacter*. In: Krieg NR, Holt JG (eds) Bergey's manual of systematic bacteriology, vol 1. Williams and Wilkins, Baltimore, pp 267–278

De Muynck C, Pereira CSS, Naessens M, Parmentier S, Soetaert W, Vandamme EJ (2007) The genus *Gluconobacter oxydans*: comprehensive overview of biochemistry and biotechnological applications. Crit Rev Biotechnol 27:147–171

Deppenmeier U, Ehrenreich A (2009) Physiology of acetic acid bacteria in light of the genome sequence of *Gluconobacter oxydans*. J Mol Microbiol Biotechnol 16:69–80

Dragosits M, Mozhayskiy V, Quinones-Soto S, Park J, Tagkopoulos I (2013) Evolutionary potential, cross-stress behavior and the genetic basis of acquired stress resistance in *Escherichia coli*. Mol Syst Biol 9:643

Efiuvwevwere BJO, Gorris LGM, Smid EJ, Kets EPW (1999) Mannitol-enhanced survival of *Lactococcus lactis* subjected to drying. Appl Microbiol Biotechnol 51:100–104

Empadinhas N, da Costa MS (2009) Diversity, distribution and biosynthesis of compatible solutes in prokaryotes. Contrib Sci 5:95–105

Galinski EA, Herzog RM (1990) The role of trehalose as a substitute for nitrogen-containing compatible solutes. Arch Microbiol 153:607–613

Groisillier A, Labourel A, Michel G, Tonon T (2014) The mannitol utilization system of the marine bacterium *Zobellia galactanivorans*. Appl Environ Microbiol. doi:10.1128/AEM.02808-14, Published online ahead of print on 29 December 2014

Gupta A, Singh VK, Qazi GN, Kumar A (2001) *Gluconobacter oxydans*: its biotechnological applications. J Mol Microbiol Biotechnol 3: 445–456

Hancock RD (2009) Recent patents on vitamin C: opportunities for crop improvement and single-step biological manufacture. Recent patents on food. Nutr Agric 1:39–49

Hanke T, Nöh K, Noack S, Polen T, Bringer S, Sahm H, Wiechert W, Bott M (2013) Combined fluxomics and transcriptomics analysis of glucose catabolism via a partially cyclic pentose phosphate pathway in *Gluconobacter oxydans* 621H. Appl Environ Microbiol 79:2336–2248

Iwamoto K, Shiraiwa Y (2005) Salt regulated mannitol metabolism in algae. Mar Biotechnol Mini Rev 7:407–415

Jennings DH (1984) Polyol metabolism in fungi. Adv Microb Physiol 25: 149–193

Kets EPW, Galinski EA, De Witt M, De Bont JAM, Heipieper H (1996) Mannitol, a novel compatible solute in *Pseudomonas putida* S12. J Bacteriol 178:6665–6670

Kets EPW (1997) Compatible solutes in lactic acid bacteria subjected to water stress. Ph.D. Thesis, Wageningen Agricultural University, Wageningen, The Netherlands

Koch AL (1997) Microbial physiology and ecology of slow growth. Microbiol Mol Biol Rev 61:305–318

Lee SY (1996) High cell density culture of *Escherichia coli*. Trends Biotechnol 14:98–105

Lewis IA, Schommer SC, Markley JL (2009) rNMR: open source software for identifying and quantifying metabolites in NMR spectra. Magn Reson Chem 47:123–126

Loos H, Krämer RH, Sprenger GA (1994) Sorbitol promotes growth of *Zymomonas mobilis* in environments with high concentrations of sugar: evidence for a physiological function of glucose-fructose oxidoreductase in osmoprotection. J Bacteriol 176:7688–7693

Luchterhand B, Fischöder T, Grimm AR, Wewetzer S, Wunderlich M, Schlepütz T, Büchs J (2015) Quantifying the sensitivity of *G. oxydans* ATCC 621H and DSM 3504 to osmotic stress triggered by soluble buffers. J Ind Microbiol Biotechnol 42:585–600

Mattey M (1992) The production of organic acids. Crit Rev Biotechnol 12:87–132

Meyer M, Schweiger P, Deppenmeier U (2013) Effects of membrane-bound glucose dehydrogenase overproduction on the respiratory chain of *Gluconobacter oxydans*. Appl Microbiol Biotechnol 97: 3457–3466

Oren A (1999) Bioenergetic aspects of halophilism. Microbiol Mol Biol Rev 63:334–348

Ortiz ME, Bleckwedel J, Raya RR, Mozzi F (2013) Biotechnological and in situ food production of polyols by lactic acid bacteria. Appl Microbiol Biotechnol 97:4713–4726

Prescott FJ, Shaw JK, Bilello P, Cragwall GO (1953) Gluconic acid and its derivatives. Ind Eng Chem 45:338–342

Pronk JT, Levering PR, Olijve W, Van Dijken JP (1989) Role of NADP dependent and quinoprotein glucose dehydrogenase in gluconic acid production by *Gluconobacter oxydans*. Enzym Microb Technol 11: 160–164

Prust C, Hoffmeister M, Liesegang H, Wiezer A, Fricke WF, Ehrenreich A, Gottschalk G, Deppenmeier U (2005) Complete genome sequence of the acetic acid bacterium *Gluconobacter oxydans*. Nat Biotechnol 23:195–200

Rabenhorst J, Gatfield I, Hilmer JM (2001) Natural, aliphatic and thiocarboxylic acids obtainable by fermentation and a microorganism therefore. Patent EP1078990

Rauch B, Pahlke J, Schweiger P, Deppenmeier U (2010) Characterization of enzymes involved in glucose and gluconate utilization in the central metabolism of *Gluconobacter oxydans*. Appl Microbiol Biotechnol 88:711–718

Richhardt J, Bringer S, Bott M (2012) Mutational analysis of the pentose phosphate and Entner-Doudoroff pathways in *Gluconobacter oxydans* reveals improved growth of a Δ*edd* Δ*eda* mutant on mannitol. Appl Environ Microbiol 78:6975–6986

Riesenberg D, Schulz V, Knorre WA, Pohl HD, Korz D, Sanders EA, Ross A, Deckwer WD (1991) High cell density cultivation of *E. coli* at controlled specific growth rate. J Biotechnol 20:17–28

Romantsov T, Stalker L, Culham DE, Wood JM (2008) Cardiolipin controls the osmotic stress response and the subcellular location of transporter ProP in *Escherichia coli*. J Biol Chem 283:12314–12323

Saha BC, Racine FM (2011) Biotechnological production of mannitol and its applications. Appl Microbiol Biotechnol 89:879–891

Sand M, Mingote AI, Santos H, Müller V, Averhoff B (2013) Mannitol, a compatible solute synthesized by *Acinetobacter baylyi* in a two-step mechanism including a salt-induced and salt-dependent mannitol-1-phosphate dehydrogenase. Environ Microbiol 15:2187–2197

Sawyer DT (1964) Metal-gluconate complexes. Chem Rev 64:633–643

Schedel M (2000) Regioselective oxidation of aminosorbitol with *Gluconobacter oxydans*, key reaction in the industrial 1-deoxynojirimycin synthesis. In: Kelly DR (ed) Biotechnology, vol 8b. Wiley-VCH, Weinheim, pp 295–308

Sévin DC, Sauer U (2014) Ubiquinone accumulation improves osmotic-stress tolerance in *Escherichia coli*. Nat Chem Biol 10:266–272

Shiloach J, Fass R (2005) Growing *E. coli* to high cell density: a historical perspective on method development. Biotechnol Adv 23:345–357

Sievers M, Swings J (2005) Family II Acetobacteriaceae. In: Garrity G, Brenner DJ, Krieg NR, Staley JT (eds) Bergey's manual of systematic bacteriology, vol 2c. Springer, New York, pp 41–95

Sootsuwan K, Thanonkeo P, Keeratirakha N, Thanonkeo S, Jaisil P, Yamada M (2013) Sorbitol required for cell growth and ethanol production by *Zymomonas mobilis* under heat, ethanol, and osmotic stresses. Biotechnol Biofuels 6:180–193

Wiegmann K, Hensler M, Wöhlbrand L, Ulbrich M, Schomburg D, Rabus R (2014) Carbohydrate catabolism in *Phaeobacter inhibens* DSM 17395, a member of the marine roseobacter clade. Appl Environ Microbiol 80:4725–4737

Winkler JD, Garcia C, Olson M, Callaway E, Kao KC (2014) Evolved osmotolerant *Escherichia coli* mutants frequently exhibit defective N-acetylglucosamine catabolism and point mutations in cell shape-regulating protein MreB. Appl Environ Microbiol 80:3729–3740

Wisselink HW, Weusthuis RA, Eggink G, Hugenholtz J, Grobben GJ (2002) Mannitol production by lactic acid bacteria: a review. Int Dairy J 12:151–161