Halotolerance of the Phototrophic Bacterium *Rhodobacter capsulatus* E1F1 Is Dependent on the Nitrogen Source

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Phototrophic growth of the moderate halotolerant *Rhodobacter capsulatus* strain E1F1 in media containing up to 0.3 M NaCl was dependent on the nitrogen source used. In these media, increased growth rates and growth levels were observed in the presence of reduced nitrogen sources such as ammonium and amino acids. When the medium contained an oxidized nitrogen source (dinitrogen or nitrate), increases in salinity severely inhibited phototrophic growth. However, the addition of glycine betaine promoted halotolerance and allowed the cells to grow in 0.2 M NaCl. Inhibition of diazotrophic growth by salinity was due to a decrease in nitrogenase activity which was no longer synthesized and reversibly inactivated, both effects being alleviated by the addition of glycine betaine. In *R. capsulatus* E1F1, inhibition of cell growth in nitrate by salt was due to a rapid inhibition of nitrate uptake, which led to a long-term decrease in nitrate reductase activity, probably caused by repression of the enzyme. Addition of glycine betaine immediately restored nitrate uptake, but the recovery of nitrate reductase activity required several hours. Neither ammonium uptake nor ammonium assimilation through the glutamine synthetase-glutamate synthase pathway was affected by NaCl.

To survive and thrive, microorganisms require a great capacity for adaptation to the continuous changes in the surrounding environment. Salinity is one of the most important factors which can affect microbial and plant growth, and the elucidation of the molecular mechanisms involved in the adaptability to salt stress is the object of current research. Although salinity and osmotic stress have been considered similar phenomena (11, 17, 21, 26), each has different components (10).

One of the mechanisms involved in the adaptation to osmotic stress consists of the cytosolic accumulation of organic or inorganic solutes (osmolytes) to restore the osmotic equilibrium (30). The first osmolytes accumulated are frequently K⁺ and L-glutamate. These will then be replaced by other compatible solutes which are able to reach high intracellular concentrations without interfering with normal metabolism or enzymatic activities (12). Most of the moderate halotolerant bacteria studied accumulate glycine betaine in response to osmotic stress (8, 16, 17), although other metabolites such as L-proline and trehalose may play important roles as osmoprotectants in some cases (8, 21).

Most phototrophic nonsulfur purple bacteria usually thrive in freshwaters and, in general, are very sensitive to low salt concentrations (16). Nevertheless, some species of *Rhodobacter*, *Rhodopseudomonas*, and *Rhodospirillum* live in marine environments with high NaCl concentrations (16). Some phototrophic bacterial species are halotolerant; i.e., they do not require NaCl for normal growth, but they tolerate relatively high concentrations of salt. Recently, a role for glycine betaine as an osmoprotectant in *Rhodobacter sphaeroides* has been reported (1).

In the present work we have studied the halotolerance of two *Rhodobacter capsulatus* strains and its effect on nitrogen

metabolism. One of these diazotrophic bacteria, *R. capsulatus* E1F1, is also able to photoassimilate nitrate (7), and ammonium produced from N₂ or nitrate is incorporated into carbon skeletons by the glutamine synthetase-glutamate synthase pathway (4). We present evidence that the halotolerance of *R. capsulatus* is dependent on the nitrogen source. Increased salinity inhibits bacterial growth on inorganic nitrogen sources by a short-term, reversible inactivation of nitrogen fixation and nitrate uptake followed by a long-term effect on nitrogenase and nitrate reductase activities, which probably affects the de novo synthesis of both enzymes.

MATERIALS AND METHODS

Organism and growth conditions. *R. capsulatus* E1F1 and B10 were cultured phototrophically in RCV medium as described previously (5), with the indicated carbon (30 mM) and nitrogen (10 mM) sources. Cell growth was monitored turbidimetrically at 680 nm, and the purity of the cultures was checked by plating them on solid agar media containing 1.8% Bacto Agar (Difco) and 0.3% yeast extract.

Cell extracts. Cells were harvested by centrifugation at 15,000 × g for 15 min and washed with 100 mM Tris-HCl buffer (pH 7.5), and depending on the analytical determination, the pellet was resuspended in either 100 mM Tris-HCl buffer (pH 8.0) or 50 mM MOPS (morpholinepropanesulfonic acid)-KOH buffer (pH 7.5) containing 2.5% (vol/vol) glycerol and 2 mM dithioerythritol. Crude extracts were obtained by ultrasonic treatment, as previously described (15).

Enzyme activities. Previously described spectrophotometric methods were used to determine the enzymatic activities of alanine dehydrogenase (EC 1.4.1.1) (3), betaine aldehyde dehydrogenase (EC 1.2.1.8) (29), glutamate synthase (EC 1.4.1.13) (5), glutamine synthetase (EC 6.3.1.2) (4), and nitrate reductase (EC 1.6.6.2) (7) in crude extracts. Nitrogenase (EC 1.18.2.1) activity was measured in whole cells by the acetylene reduction technique as described by Zumft and Castillo (32). One unit of activity is the amount of enzyme that transforms 1 μmol of substrate per min, and specific activity is expressed as units per milligram of protein. Nitrate, nitrite, or ammonium uptake was determined as previously described (6).

Analytical procedures. Internal amino acids were determined by high-pressure liquid chromatography as described by Márquez et al. (22). Lipids and total carbohydrates were estimated according to Zöllner and Kirsch (31) and Dubois et al. (9), respectively. Protein was routinely estimated according to Bradford (2), using ovalbumin as the standard. [³⁵S]methionine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Fernandes et al. (10). Monoclonal antibodies for enzyme-linked immunosorbent assay (ELISA), against the α subunit of component I of *Klebsiella pneumoniae*

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FIG. 1. Effect of salinity on growth of *R. capsulatus* E1F1. Cells were grown phototrophically in RCV media with malate and ammonium as carbon and nitrogen sources, respectively, without additions (●) or in the presence of 0.1 (○), 0.2 (△), or 0.3 (▽) M NaCl. Growth was measured at 680 nm.

nitrogenase, were kindly supplied by N. J. Brewin (John Innes Institute, Norwich, United Kingdom).

RESULTS

Effect of increasing osmolarity on bacterial growth. Figure 1 shows the growth curves obtained with *R. capsulatus* E1F1 cells cultured anaerobically in the light in media containing malate and ammonium as the carbon and nitrogen sources with increasing NaCl concentrations. Growth profiles were very similar in the RCV normal medium (50 mM NaCl) and with NaCl up to a concentration of 0.2 M. In medium with 0.3 M NaCl, a longer lag phase was observed and growth took place at a lower rate. Cell cultures showed similar pH values under the different growth conditions tested. Figure 2 summarizes the results obtained when *R. capsulatus* E1F1 was cultured phototrophically in media with oxidized and reduced nitrogen sources in the presence of different NaCl concentrations. In media containing ammonium or amino acids, similar growth yields were observed in cells cultured with NaCl up to a concentration of 0.2 M. The generation time was always about 3 to 4 h. Both parameters, in particular, the generation times (which were increased fivefold), were affected by 0.3 M salt. No growth was observed above this value (data not shown). By contrast, when the bacteria were grown in media with N_2 or nitrate, growth yields and growth rates were severely inhibited by NaCl at concentrations below 0.2 M (Fig. 2). The total amounts of sugar and lipids did not change under the different growth conditions studied.

Similar results were obtained when the other bacterial strain studied, *R. capsulatus* B10, was grown on N_2 or reduced nitrogen sources in the presence of NaCl.

Because of the ability of *R. capsulatus* E1F1 to use both nitrate and N_2 as a nitrogen source, we mainly used this strain

FIG. 2. Effect of NaCl and glycine betaine on phototrophic growth of *R. capsulatus* E1F1. Cells were cultured anaerobically under light in RCV media with the indicated nitrogen sources without additions (A) or with 0.1 M NaCl (B), 0.2 M NaCl (C), 0.3 M NaCl (D), or 0.2 M NaCl and 10 mM glycine betaine (E). Except where indicated (Pyr/ammonium), malate was the carbon source. Each growth condition was tested at least four times, and values of A_{680} represent the average of the maximal values observed during cell growth.

to study the effect of nitrogen source on bacterial halotolerance.

Effect of glycine betaine on bacterial growth in the presence of NaCl. The effect of glycine betaine on *R. capsulatus* E1F1 growth is presented in Fig. 2 and 3. Growth with N_2 or nitrate in the presence of 0.2 M NaCl was only achieved when the medium contained 10 mM glycine betaine. The addition of the osmolyte to cultures with N_2 or nitrate and 0.2 M NaCl (which hence showed no growth) allowed the cells to grow after a lag phase of about 24 h with a much lower growth rate than that observed when the media contained glycine betaine from the beginning of the growth period (Fig. 3). After 5 days of growth, maximal A_{680} values were 0.7 and 0.3 for cells grown with nitrate and dinitrogen, respectively. It is worth noting that the bacterial strains tested were unable to use glycine betaine as a nitrogen source under the different growth conditions (data not shown). The addition of choline to the cultures containing N_2 or nitrate and 0.2 M NaCl was without effect (data not shown). L-Proline and L-glutamate also alleviated the osmotic shock but to a lesser extent than glycine betaine.

Effect of NaCl and glycine betaine on nitrate assimilation. When the culture medium containing *R. capsulatus* E1F1 cells growing phototrophically with nitrate as the sole nitrogen source was adjusted to 0.2 M NaCl, an immediate cessation of nitrate uptake occurred (Fig. 4B). This was followed by a slow

FIG. 3. Protection by glycine betaine from the effect of salinity on growth of *R. capsulatus* E1F1. Cells were grown under phototrophic conditions in RCV media with 30 mM malate as carbon source and 10 mM nitrate (A) or dissolved N_2 (B) as nitrogen source. ●, no addition; open symbols, cultures with 0.2 M NaCl; △, cultures plus 10 mM glycine betaine from time zero; ○, cultures made 10 mM in glycine betaine at the time indicated by the arrow; □, cultures with NaCl and without glycine betaine.

and significant decrease in nitrate reductase activity (Fig. 4A) and, subsequently, in the nitrite concentration in the medium (Fig. 4C). Under such conditions, cell growth stopped immediately (not shown). If the culture contained both 0.2 M NaCl and 10 mM glycine betaine, cell growth did not stop and the nitrate uptake resumed at the same rate observed before additions (Fig. 4B). A decrease in nitrate reductase activity was detected after NaCl addition, even in the presence of glycine betaine, but 3 h after the addition, the original activity was completely restored (Fig. 4A). The addition of NaCl affected nitrite excretion, which was reversed to nitrite uptake after the culture was made 0.2 M in NaCl (Fig. 4C). The presence of glycine betaine did not affect the uptake of nitrite (Fig. 4C). Intracellular amino acid concentrations, including L-proline, remained unaltered after either NaCl or glycine betaine addition with the exception of L-glutamate, which showed a 20% increase in the presence of salt. Nevertheless, intracellular L-glutamate levels returned to the basal values after 30 min of NaCl treatment (results not shown).

Modulation of nitrogenase activity by NaCl and glycine betaine. NaCl inhibited nitrogen fixation in diazotrophic cultures of *R. capsulatus* E1F1 by affecting nitrogenase synthesis and activity. When nitrogen-fixing cultures were made 0.2 M in NaCl, ethylene production was drastically decreased (Fig. 5A), the inhibitory effect being observed for more than 20 h. However, the original nitrogenase activity could be immediately restored by the addition of glycine betaine to the same cells (Fig. 5A). Similar results were obtained in the presence of

FIG. 4. Effect of NaCl on nitrate uptake by *R. capsulatus* E1F1. Cultures containing cells growing phototrophically with nitrate as nitrogen source were made, at the time indicated by the arrow, 0.2 M in NaCl without (○) or with (▽) glycine betaine to a final concentration of 10 mM. ●, control without additions. In these cultures, nitrate reductase activity (A), nitrate (B), and nitrite (C) in the medium were determined at the indicated times.

chloramphenicol (20 μ g/ml of culture). If diazotrophic cells were treated with the osmolyte prior to NaCl addition, only a 35% inhibition of nitrogenase activity took place (Fig. 5B).

Nitrogenase activity could not be detected in cells growing with 0.2 M NaCl regardless of the nitrogen source used for growth, unless glycine betaine was also present (Table 1). Nitrogenase proteins were absent in *R. capsulatus* extracts from cells cultured with NaCl, as deduced from [35 S]methionine-SDS-PAGE and ELISA experiments (data not shown).

When glycine betaine was added to cultures with L-glutamate and 0.2 M NaCl which lacked nitrogenase activity, ethylene production was slowly restored at a rate dependent upon the concentration of the osmolyte (Fig. 6). The activity was not restored in the presence of chloramphenicol. A slight activating effect was also observed when the culture was made 10 mM in choline (Fig. 6). Cell extracts of *R. capsulatus* E1F1 cultured

FIG. 5. Effect of NaCl on nitrogenase activity of *R. capsulatus* E1F1. Nitrogenase activity was determined in cells which had been growing with malate-glutamate in the absence of salt. At the times indicated by the arrows, the cultures were made 0.2 M in NaCl (A, arrow 1) and after nitrogenase was inhibited, glycine betaine was added to a final concentration of 10 mM (A, arrow 2). The order of these additions was inverted in panel B: arrow 2, addition of glycine betaine to a final concentration of 10 mM; arrow 1, addition of NaCl to a final concentration of 0.2 M.

with 10 mM choline exhibited an NAD^+ -dependent betaine aldehyde dehydrogenase activity (15 mU/mg of protein). Reduction of NAD^+ by these extracts was coupled to choline consumption, probably due to a choline oxidase activity which yielded betaine aldehyde (data not shown).

Effect of NaCl on enzymatic activities related to ammonium metabolism. To check the effect of salt shock on the metabolism of ammonium and amino acids, *R. capsulatus* E1F1 cells were cultured in malate-glutamate or malate-ammonium. The effect of NaCl on ammonium uptake was previously studied, with negative results (data not shown). The levels of glutamine

FIG. 6. Effect of NaCl on nitrogenase synthesis in *R. capsulatus* E1F1. Cells were cultured with malate-glutamate in the presence of 0.2 M NaCl. At the time indicated by the arrow, different cultures were made 1 (▽) or 10 (Δ) mM in glycine betaine or 10 mM (○) in choline. Nitrogenase activity was measured in whole cells for the time indicated.

synthetase and glutamate synthase activities, which catalyze the main ammonium-assimilating pathway in these cells, were not significantly affected by 0.2 M NaCl (data not shown). However, a decrease of about 60% in alanine dehydrogenase activity, a bifunctional enzyme which participates in the assimilation of ammonium and in the catabolism of L-alanine and L-glutamate in *R. capsulatus*, could be observed.

DISCUSSION

In this work we have investigated the relationship between the halotolerance of different *R. capsulatus* strains and the nitrogen source used under light and anaerobiosis. High saline concentrations inhibited phototrophic growth, but halotolerance appeared when the bacterium was supplied with reduced nitrogen sources such as ammonium or amino acids, contrary to results reported in *R. sphaeroides* (1). This bacterium synthesizes glycine betaine as the main osmolyte, but the intracellular L-glutamate concentration is also raised in response to increased osmolarity (1). In *R. capsulatus* E1F1 and B10, exogenous glycine betaine not only increased halotolerance but enabled the cells to grow with oxidized nitrogen sources in the presence of salt. As in *R. sphaeroides*, L-glutamate was the only amino acid which accumulated after the addition of 0.2 M NaCl, although in the case of the *R. capsulatus* strains studied, the L-glutamate concentration inside the cells dropped again after 30 min of NaCl treatment.

Csonka and Hanson (8) have demonstrated a general osmoregulatory mechanism in bacteria which consists of a two-step response: a rapid accumulation of K^+ and net synthesis of L-glutamate followed by the accumulation of other osmoprotectants. Thus, Madkour et al. (21) reported that different diazotrophic bacteria (*Azotobacter chroococcum*, *Azospirillum*

TABLE 1. Effect of NaCl on nitrogenase activity of *R. capsulatus* E1F1^a

Culture	Nitrogen source	Glycine betaine (mM)	NaCl (M)	N_2 ase activity (%) ^b
I	L-Glutamate			100
II	L-Glutamate		0.2	ND ^c
III	L-Glutamate	10		105
IV	L-Glutamate	10	0.2	90
V	N_2			73
VI	N_2		0.2	ND
VII	N_2	10		75
VIII	N_2	10	0.2	70

^a Cells were cultured anaerobically under light with malate as carbon source and dissolved N_2 or 10 mM L-glutamate as nitrogen source in the presence and in the absence of glycine betaine and/or NaCl.

^b Nitrogenase activity values were the maximal observed during cell growth and were relative to the control culture with L-glutamate without glycine betaine and salt. Activity of 100% corresponds to 65 mU/mg of protein.

^c ND, not detectable.

brasile and *K. pneumoniae*) accumulate potassium ions and L-glutamate and, after induction of the corresponding enzymatic systems, produce L-proline (*A. brasile*), trehalose (*A. chroococcum*), or both (*K. pneumoniae*). Another osmolyte is glycine betaine, which is synthesized by an aerobic pathway, although most bacteria depend on exogenous betaine present in natural environments (8).

In *Rhizobium meliloti*, a pathway for the synthesis and degradation of glycine betaine from choline has been described (29). This pathway is under osmotic control, and the rate-limiting step is the demethylation of glycine betaine (29). In *R. capsulatus* E1F1, NAD⁺ dehydrogenase activity is dependent on choline. This activity has been measured in extracts from cells growing in media with a high choline concentration. This reduction could be due to the coupling of the two first enzymes of the pathway described by Smith et al. (29). Nevertheless, choline did not increase osmotolerance of *R. capsulatus* E1F1 under phototrophic conditions (light and anaerobiosis), as the first enzyme is an oxidase which requires significant amounts of O₂ in vivo to be fully active. In *R. sphaeroides*, choline only acts as an osmoprotectant in aerobic, darkened cells (1). This effect could not be proved in *R. capsulatus* because in this bacterium nitrogen fixation, which was strongly inhibited by salt shock, requires anaerobic conditions of growth (20, 25). On the other hand, strain E1F1 only assimilates nitrate under light and anaerobic conditions (7).

Little is known about the influence of nitrogen sources on bacterial growth in hypersaline environments. In this work we have used *R. capsulatus* E1F1, which is capable of photoassimilating nitrate in addition to fixing dinitrogen. In the case of N₂ fixation, salinity strongly inactivated nitrogenase by a double effect on the synthesis and activity of the enzyme. The short-term effect on nitrogenase by NaCl was rapidly alleviated by glycine betaine in a process unaffected by the presence of chloramphenicol, which suggests that salt shock affected the activity of the enzyme and that de novo protein synthesis was not involved in this process. Nevertheless, the time required for both inactivation and reactivation of nitrogen fixation is considerably longer than that required for ammonium switch-off (32). In the cyanobacterium *Anabaena* sp., nitrogen fixation is strongly inhibited by moderate NaCl concentrations and the bacterium responds to saline stress by synthesizing new proteins and repressing the synthesis of others (10).

In cyanobacteria, nitrogenase inactivation by salt has been explained as a deviation of cellular energy to osmoregulatory processes, in particular, to the efflux of Na⁺ (10). This may also occur in the short-term nitrogenase inhibition observed in *R. capsulatus* E1F1. Glycine betaine could act by restoring the osmotic balance over the membrane. Nevertheless, a direct specific protective effect of the osmolyte on nitrogenase against high NaCl concentration cannot be dismissed (1, 30).

Nitrogen fixation undergoes a second, long-term inhibition by NaCl which is related to nitrogenase synthesis. The long-term effect was observed in cells growing in media containing 0.2 M NaCl. After these cells were passed into an NaCl-free medium or glycine betaine was added, the recovery of nitrogen fixation activity required a long period of time (more than 10 h). As was proposed by Higgins et al. (13), NaCl provoked repression of enzymes. The effect of the osmolyte could be to activate nitrogenase induction and prevent the inactivation of the newly synthesized enzyme. In *R. capsulatus* E1F1, this conclusion is supported by the effect of chloramphenicol, which prevents nitrogenase recovery after the glycine betaine addition to cells growing in media with 0.2 M NaCl. The effect of chloramphenicol suggests that de novo protein synthesis was involved in this case. This also agrees with the results obtained

in [³⁵S]methionine-SDS-PAGE and ELISA experiments. On the other hand, the time required for recovering nitrogenase activity in media with NaCl after the glycine betaine addition was similar to that observed for inducing nitrogenase activity in washed *R. capsulatus* E1F1 cells cultured with glutamine, which is known to repress *nif* genes (18, 27).

In the case of nitrate photoassimilation, the most drastic effect caused by NaCl was the complete inhibition of nitrate uptake, which was followed by a slow and continuous decrease in nitrate reductase activity. In nitrate-assimilating *R. capsulatus* E1F1 cells, some nitrite formed from nitrate reduction appeared in the medium in a nonstoichiometric amount (7). After the NaCl treatment, nitrite excretion was shifted to nitrite uptake as a logical consequence of the inhibition of nitrate uptake, which resulted in a decreased nitrate reduction inside the cells. Nitrate uptake and nitrite uptake in *R. capsulatus* E1F1 require metabolic energy and take place by different transport systems (6). This agrees with the results discussed above showing that nitrate uptake was stopped by 0.2 M NaCl whereas nitrite uptake was not. It may be suggested, therefore, that there is a direct effect of NaCl on the nitrate transporter instead of an indirect effect on energy supply which would also affect nitrite uptake. Since the KCl addition to a final concentration of 0.2 M showed the same effect, a competition between NO₃⁻ and Cl⁻ ions for the membrane transporter might also be suggested. Inhibition of nitrate uptake by chloride has been found in green algae (1a).

The effect of NaCl on nitrate reductase levels is similar to that observed after ammonium addition to nitrate-growing cells: ammonium strongly inhibits nitrate uptake and the decrease of intracellular nitrate represses nitrate reductase (23, 24). After the NaCl addition, the nitrate uptake was strongly inhibited, and a drop in intracellular nitrate would repress nitrate reductase. Protection by glycine betaine may be explained by taking into account that the nitrate uptake was restored at the same rate once osmolyte was present in the culture. A transient efflux of nitrate prior to resumption of nitrate uptake could be due to the coupling of the glycine betaine entry with the K⁺ ejection that can be accompanied by NO₃⁻ release. Once the glycine betaine transport system was induced, nitrate uptake and nitrate reductase levels reached the control values observed in cultures without NaCl. Although nitrate has been described to be an osmoprotectant in some cyanobacteria (10, 28), this is obviously not the case in *R. capsulatus* E1F1 because of the inhibition of nitrate transport by NaCl. Respiratory nitrate reductase from *Escherichia coli* is also inactivated by NaCl in a process mediated by intracellular K⁺ which can be reversed by glycine betaine (11).

Osmotic adaptability requires some genes regulated by osmoregulation. Thus, osmoprotection by glycine betaine in *E. coli* is linked to the ProP and ProU transport systems, whereas uptake of K⁺ requires the Trk and Kdp systems (8, 12). Among the proteins coded by osmoregulatory genes, there would be enzymes of L-glutamate metabolism, since this amino acid is often involved in osmoprotection. From our results, it cannot be concluded that glutamate synthase is involved in salt tolerance in *R. capsulatus* E1F1 as neither glutamine synthetase nor glutamate synthase activities change in response to NaCl treatment. Nevertheless, the glutamate synthase dimer suffers an aggregation-disaggregation process dependent on the ionic strength which has been related to kinetic regulation of the enzyme by allosteric effects (14). This may be the only regulatory process which would link increasing salinity (with a raise in intracellular K⁺) and glutamate synthase activity. Thus, a transient activation of GOGAT would cause the slight oscillation in intracellular L-glutamate observed after salt shock.

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