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A Prominent Role for Glucosylglycerol in the Adaptation of *Pseudomonas mendocina* SKB70 to Osmotic Stress

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The mechanism of osmoadaptation in a salt-tolerant (1.2 M NaCl) bacterial isolate identified as *Pseudomonas mendocina* (N. J. Palleroni, M. Doudoroff, R. Y. Stanier, R. E. Solanes, and R. Mandel, J. Gen. Microbiol. 60:215–231, 1970) was investigated. In response to osmotic stress, this species accumulated a number of compatible solutes, the intracellular levels of which depended on both the osmolarity and the ionic composition of the growth medium. Glucosylglycerol [α-D-glucopyranosyl-α-(1→2)-glycerol], *N*-acetylglutaminylglutamine amide, and L-α-glutamate were the major compatible solutes accumulated via de novo biosynthesis. Trehalose was also accumulated, but only in cells grown in the presence of high concentrations of sulfate or phosphate ions. Glycine betaine was accumulated only when supplied exogenously to cells grown at high osmolarity, and its accumulation caused a significant depletion of the intracellular pools of glucosylglycerol and glutamate. Glucosylglycerol was also found to accumulate in the type strains of *P. mendocina* and *P. pseudoalcaligenes*. This is the first report demonstrating the pivotal role of glucosylglycerol in osmoadaptation in a nonphotosynthetic microorganism.

Microorganisms survive and proliferate in environments of varied ionic composition and salinity ranging from freshwater to hypersaline habitats. To grow in an osmotically stressful environment, a bacterial cell must establish and maintain its internal pressure above that of its surrounding medium. This is commonly achieved through the accumulation of osmotically active solutes (osmolytes) in the cytosol of prokaryotic and eukaryotic cells (7, 13, 23, 37). Because they do not interfere with macromolecular function and maintain physiological processes at high intracellular concentrations, osmolytes are also termed compatible solutes. Potassium and glutamate are ubiquitous bacterial osmolytes which are usually accumulated together as mutual counterions. Most other osmolytes are low-molecular-weight organic compounds which share the following features: high solubility in water, a neutral net electric charge at a physiological pH, and a lack of toxicity toward enzymes when assayed in vitro at high but physiologically relevant concentrations (8, 11, 35, 48).

Although they are accumulated to significant levels, some osmolytes have escaped detection by usual chemical methods because they lacked a reactive group (39, 42) or because of specific deficiencies of the analytical methods employed (37). Natural-abundance ^{13}C nuclear magnetic resonance (NMR) spectroscopy is a nondestructive method enabling the detection of all organic compatible solutes that accumulate to significant levels in bacteria, in plants, or in animal tissues. A unique attribute of NMR spectroscopy is its noninvasiveness, which sometimes allows the detection of osmolytes directly in situ, in intact bacterial cells, in organelles, or in tissue fragments (8, 13, 25, 39). Fast atom bombardment mass spectrometry is also widely used in the study of osmoregulation and is especially useful when the biological material is available in limited amounts (36, 37). Interestingly, NMR spectroscopy and fast atom bombardment mass spectrometry have recently contributed to the discovery of a series of new osmolytes including sugars and amino acid derivatives from a wide variety of bacterial and plant species (4, 8, 11, 37).

Species of the genus *Pseudomonas* are metabolically very versatile and are encountered in most natural environments, from freshwater to hypersaline habitats (17). They are intensively studied because of their primary importance in medicine, phytopathology, food spoilage, biological control, and environmental research (17, 29, 30, 45). Species such as *Pseudomonas putida*, *P. stutzeri*, and *P. mendocina* have a particularly high potential for bioremediation of contaminated soils and waters because they can detoxify recalcitrant pollutants such as organomercurials, pesticides, and halogenated aromatic compounds found in crude oil and petroleum (45). However, efficient biodegradation of these compounds in polluted environments is often limited by other abiotic stresses such as pH, high temperature, desiccation, and salinity (45). In spite of the potential uses of these organisms, little is known about the mechanisms that allow beneficial *Pseudomonas* spp. to thrive in desiccated soils or in high-osmolarity media such as sewage, seawater, or hypersaline lakes (1, 9, 38). It has been shown, however, that the osmoprotectant glycine betaine enhances the survival of *P. aeruginosa* ATCC 27853 in seawater (1). Also, betaine strongly stimulates the biosynthesis of cyclopropane fatty acids, which in turn increase membrane stability at high osmolarity in extremely salt-tolerant *P. halosaccharolytica* CCM 2851 (27). Further investigation of the mechanisms of osmoregulation in *Pseudomonas* spp. is obviously warranted prior to genetically engineering salinity tolerance in strains that hold promise for environmental research and biotechnology.

Here, we report the identity of osmolytes accumulated by a particularly salt-tolerant strain of *P. mendocina* and provide evidence for the modulation of the intracellular levels of these

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osmolytes by environmental factors in addition to high osmolarity.

MATERIALS AND METHODS

Chemicals. All reagents used in this study were analytical grade or best grade commercially available. α-Glucosidase type III (Maltase; α-D-glucoside glucohydrolase [EC 3.2.1.20]) from baker's yeast and β-D-glucosidase glucohydrolase (EC 3.2.1.21) from almond were obtained from Sigma Chemical Co. (St. Louis, Mo.).

Bacterial strains and media. Type strains of *P. mendocina* (ATCC 25411) and *P. pseudoalcaligenes* (ATCC 17440) (30) were obtained from the American Type Culture Collection (Rockville, Md.). *P. stutzeri* JM300 (5) was obtained from A. T. Abdelal (Georgia State University). *P. mendocina* SKB70 was purified as a salt-tolerant bacterium from a culture of *Rhizobium meliloti* ABS7 isolated from a root nodule of a drought-tolerant alfalfa (*Medicago ciliaris* L.) (2). It was identified to the species level after analysis of its fatty acid content and nutritional characteristics (see below).

All strains were maintained on solid Luria-Bertani medium (26). Proline, glycine betaine, and sucrose were sterilized by filtration before addition to L-malate–Casamino Acids (MCAA) medium. Medium osmolarity was measured by using a Wescor 5500 vapor pressure osmometer. Cultures for ^{13}C NMR spectroscopy were grown in MCAA medium (39), with additions as noted. Bacteria were first grown overnight in Luria-Bertani medium, subcultured in MCAA, and used to inoculate (5%) 1-liter cultures in MCAA. In some experiments lactate-aspartate (LAS) medium was used (39). All cultures were grown aerobically at 32°C.

Identification of *P. mendocina* SKB70. Strain SKB70 was identified by cellular fatty acid analysis using the Microbial Identification System software package (MIDI Inc., Newark, Del.), conducted at Analytical Systems Inc. (Essex Junction, Vt.) and by carbon utilization tests using the Biolog GN identification test panel, conducted at Biolog Inc. (Hayward, Calif.) and Analytical Systems Inc. Geraniol utilization was tested in a plate assay by the method of Palleroni and Doudoroff (31). *P. mendocina* ATCC 25411 and *P. pseudoalcaligenes* ATCC 17440 were included in this assay as positive- and negative-control strains, respectively (30).

Sample preparation for NMR spectroscopy. Unless otherwise indicated, cultures were harvested at late log phase to early stationary phase. Samples for NMR spectroscopy were prepared as described by Smith and Smith (39), except that the combined volume of the perchloric acid extracts was 14 ml. Natural-abundance ^{13}C NMR spectra were obtained at 75.58 MHz by using a General Electric Ω-300 spectrometer and 1,024 acquisitions. Other NMR parameters were as described previously (9). The compounds in the extracts were identified by comparing the chemical shifts in the spectrum with those of authentic compounds (L-glutamate, *N*-acetylglutaminylglutamine amide [NAGGN], α,α-D-trehalose, L-proline, and glycine betaine) or published values for glucosylglycerol (4, 28, 47). Osmolyte concentrations were determined by comparison of the peak heights with an internal standard (50 mM alanine) and were verified in some cases by high-pressure liquid chromatography (HPLC) or by amino acid analysis. All experiments were run at least in duplicate, generally with less than 15% variation between samples. The intracellular osmolyte content is expressed in nanomoles per milligram of cellular protein as determined by the method of Lowry et al. (24).

Identification of glucosylglycerol. By using both physical and chemical techniques, glucosylglycerol was identified in partially purified cellular extracts of salt-stressed *P. mendocina* SKB70. Uncharged osmolytes (glucosylglycerol and NAGGN) were purified from the other components by passage over a 6-ml AG 501-X8D mixed-bed resin (Bio-Rad Laboratories, Hercules, Calif.). The deionized sample was lyophilized, resuspended in 0.5 ml of distilled water, and subjected to mass spectral analysis, HPLC, and mineral acid and enzymatic hydrolyses. Purity was ascertained by ^{13}C NMR spectroscopy.

Mass spectral analysis. The molecular masses of NAGGN and glucosylglycerol were determined from a deionized sample on a ZA6-H5-2F mass spectrometer (VG Analytical, Wythenshawe, United Kingdom). An 8-KeV xenon beam was used for fast atom bombardment. The fast atom bombardment matrix was dithiothreitol-dithioerythritol (3:1), doped with $LiHCO_3$.

HPLC analysis. Deionized samples were chromatographed on an Econosphere NH2 column (250 by 4.6 mm) with 5-μm packing (Alltech Associates, Deerfield, Ill.). The chromatograph consisted of a metering minipump (LDC/Milton Roy, Riviera Beach, Fla.), an Applied Sciences pulse damper (Alltech Associates), a LoPulse pulse damper (Scientific Systems, Inc., State College, Pa.), and a injection valve (Cotati, Calif.) Rheodyne with a 20-μl loop. An evaporative light scattering detector (Applied Chromatography Systems, Ltd.) and a Hewlett-Packard 3390A integrator were used for quantitation. The mobile phase was acetonitrile-water (80:20), and the flow rate was 2 ml/min. The retention times for NAGGN, glucosylglycerol, α-D-glucose, and α,α-D-trehalose were 3.7, 5.9, 6.7, and 15.8 min, respectively.

Mineral acid and enzymatic hydrolysis of glucosylglycerol. Deionized samples were treated with 1 N HCl at 100°C for either 40 min or 2 h, and the hydrolytic products were analyzed by HPLC and ^{13}C NMR spectroscopy. Standard solutions (0.5 to 10 mg/ml) of α-D-glucose and glycerol, the potential hydrolytic products of glucosylglycerol, were prepared both in water and in 0.2 N HCl. HPLC analysis revealed that glycerol yielded a broad, short peak (ca. 2.8 min), which was not quantitated reproducibly. Consequently, hydrolyzed glucosylglycerol was quantitated by measuring liberated glucose.

Deionized samples were also subjected to enzymatic hydrolysis by α- and β-D-glucosidases to determine the structural configuration of the glucosidic linkage in glucosylglycerol. Approximately 20 μmol of glucosylglycerol was incubated with 10 U of either enzyme in 75 mM sodium acetate for 0.5 to 25 h at room temperature. At each time point, 50 μl of the reaction mixture was removed, added to 200 μl of 0.2 N HCl to stop the reaction, and analyzed by HPLC.

Osmotic downshock experiment. A 200-ml culture was grown overnight in MCAA medium plus 0.8 M NaCl, divided into two portions, and harvested by centrifugation. The supernatants were discarded, and one pellet was resuspended in the mineral base of MCAA medium (no malate or Casamino Acids) plus 0.8 M NaCl and the other was resuspended in salts plus 0.2 M NaCl. The samples were incubated at room temperature for 30 min, enumerated, and then harvested by centrifugation. The supernatants were deionized and lyophilized, and the pellets were extracted twice with 15 ml of 70% ethanol. The glucosylglycerol contents of both the supernatant and the extracted pellet were determined by HPLC.

RESULTS

Identification of isolate SKB70. *P. mendocina* SKB70 was identified by analysis of its fatty acid content and by a number of physiological tests. The whole-cell fatty acid profile yielded similarity indices of 0.81, 0.44, and 0.25 for *P. pseudoalcaligenes*, *P. mendocina*, and *P. stutzeri*, respectively. Thus, on this

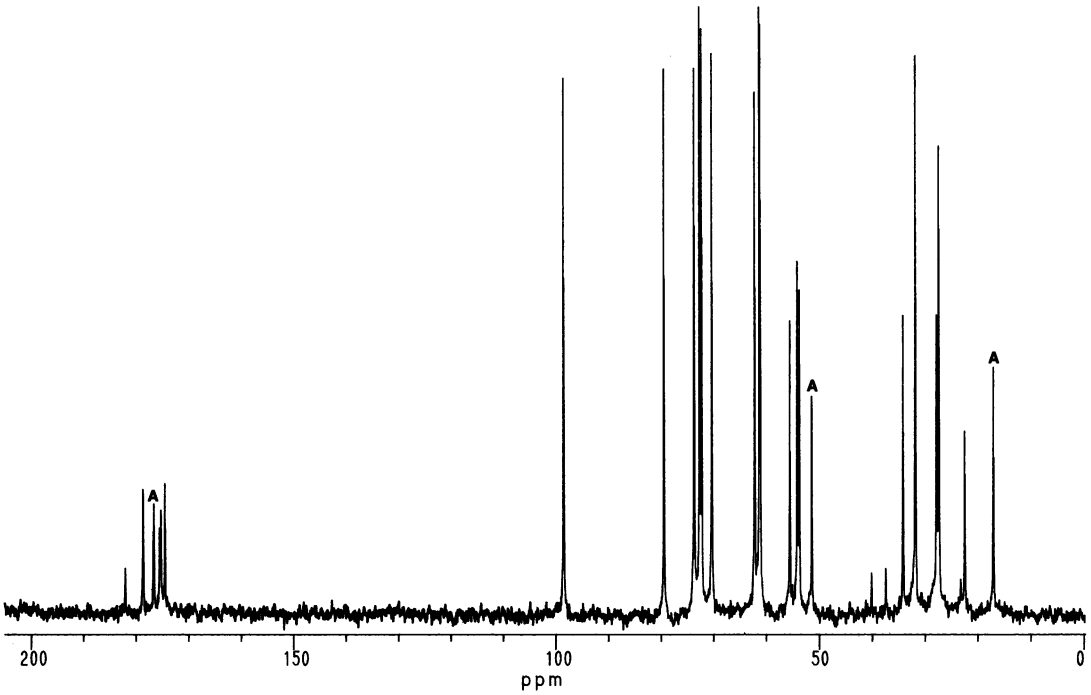

FIG. 1. Natural-abundance ^{13}C NMR spectrum of a perchloric acid extract of *P. mendocina* SKB70 grown in 0.8 M NaCl–MCAA medium. Resonances at 176.7, 51.5, and 17.1 ppm (A) arise from alanine which was added as the internal concentration standard (50 mM). Resonances at 182.1, 175.5, 55.6, 34.2, and 27.8 ppm are from L-α-glutamate. Signals at 178.7, 176.8, 175.4, 174.6, 54.2, 31.9, 27.4, and 22.6 arise from NAGGN (39). The nine resonances between 61.2 and 98.6 ppm are from glucosylglycerol.

basis isolate SKB70 appeared to be a member of rRNA group I of the genus *Pseudomonas* (30, 32). The Biolog GN nutritional test yielded a similarity index of 0.83 for *P. mendocina*. Similarity indices for *P. pseudoalcaligenes*, *P. stutzeri*, and all other bacterial species in the data bank were not taxonomically significant ($P \leq 0.05$). Thus, these results confirm that strain SKB70 is a *Pseudomonas* species (30). However, in contrast to the fatty acid analysis, the nutritional analysis strongly suggests that the species is more likely *P. mendocina* rather than the closely related species *P. pseudoalcaligenes* (14, 30, 32). Additional characteristics confirming this assignment were the yellow cellular pigmentation of the strain, a distinctive feature of *P. mendocina* (32), and its ability to grow at the expense of geraniol, a property that is strictly restricted to the species *P. aeruginosa* and *P. mendocina* (30).

Compatible solutes of *P. mendocina* SKB70. *P. mendocina* SKB70 was found to be particularly salt tolerant compared with other *Pseudomonas* species investigated in this laboratory (9, 39). For example, its doubling time was 1 h in MCAA medium and only 5.1 h in MCAA plus 1.0 M NaCl. Furthermore, growth was observed in MCAA medium containing as much as 1.2 M NaCl or KCl. In contrast, the other pseudomonads studied, *P. aeruginosa* PAO1, *P. fluorescens*, and *P. stutzeri* JM300, could not be grown at 1 M NaCl (9, 38a, 39). The striking salt tolerance of strain SKB70 led us to investigate the mechanism of osmotic adaptation in this species in detail.

Natural-abundance ^{13}C NMR spectroscopy was used to identify all organic osmolytes accumulated to significant levels in *P. mendocina* SKB70 in response to salt stress. The spectrum (Fig. 1) of a perchloric acid extract of cells grown in 0.8 M NaCl–MCAA medium included resonances that arose from L-α-glutamate (182.1, 175.5, 55.6, 34.2, and 27.8 ppm) and the dipeptide NAGGN (178.7, 176.8, 175.4, 174.6, 54.2, 31.9, 27.4, and 22.6 ppm) (39). There were also nine unidentified resonances (98.6, 79.5, 73.8, 72.7, 72.4, 70.4, 62.3, 61.3, and 61.2 ppm) that were highly reminiscent of those of *O*-α-D-glucopyranosyl-(1→2)-glycerol (glucosylglycerol), a heteroside that accumulates in salt-stressed marine cyanobacteria (4, 28, 33, 47). These nine resonances were the most intense of the spectrum, suggesting that the putative heteroside is the major organic solute accumulated in *P. mendocina* SKB70 grown at 0.8 M NaCl (Fig. 1). The ^{13}C NMR spectrum of an ethanolic extract from a duplicate culture showed exactly the same resonances of equal intensities, indicating that extraction with perchloric acid had not altered the osmolyte composition of the first culture.

Structural identification of glucosylglycerol. To verify the identity of glucosylglycerol, the cellular extract was deionized and subjected to fast atom bombardment mass spectrometry. The spectrum yielded two molecular ion masses of 322 and 261 which correspond to $(NAGGN \text{ plus } Li)^+$ (39) and $(GG \text{ plus } Li)^+$, respectively. The spectrum also showed two major fragments with masses of 161 and 99 which corresponded to $(glucose \text{ plus } Li)^+$ and $(glycerol \text{ plus } Li)^+$, respectively. These results are consistent with the NMR spectral analysis which indictated the presence of glucosylglycerol and NAGGN in salt-stressed *P. mendocina* SKB70.

A portion of the cellular extract was also subjected to a mild acid treatment. HPLC analysis of the hydrolytic products revealed that about 70% of the putative glucosylglycerol pool was hydrolyzed to glucose and glycerol after a 40-min treatment and that the hydrolysis was complete after 2 h (data not shown). A ^{13}C NMR spectrum of the hydrolyzed sample confirmed these results. The spectra of the hydrolytic products were identical to those of authentic D-glucose and glycerol (data not shown), providing further evidence for the occur-

TABLE 1. Osmolytes detected in cellular extracts of *P. mendocina* SKB70 grown at high osmolarity

Medium (MPa)	Amt[a] (nmol/mg of protein) of:			
	Glutamate	NAGGN	GG	Trehalose
MCAA (0.1)	30	UD	UD	UD
+ 0.8 M NaCl (3.8)	400	420	700	UD
+ 0.8 M KCl (3.5)	410	460	780	UD
+ 0.7 M K_2SO_4 (3.6)	370	380	420	170
+ 0.7 M Na_2SO_4 (3.6)	660	590	780	230
+ 0.5 M K_2HPO_4 (2.6)	340	300	UD	220
+ 0.6 M sucrose[b] (1.7)	40	50	160	UD
LAS + 0.8 M NaCl (3.8)	500	430	770	UD

[a] Quantitated by ^{13}C NMR spectroscopy as described in Materials and Methods. GG, glucosylglycerol; UD, undetectable (<20 nmol/mg of protein).

[b] To remove extracellular sucrose, the pellet was washed in 0.45 M NaCl (iso-osmotic to 0.6 M sucrose). Intracellular sucrose amounted to 230 nmol/mg of protein.

rence of glucosylglycerol in stressed cultures of *P. mendocina* SKB70.

To determine the configuration of the glycosidic linkage between the glucopyranosyl and glycerol moieties of glucosylglycerol, the deionized extract was subjected to cleavage by α- and β-glucosidases. HPLC analysis of the reaction products revealed that over 80% of the heteroside was hydrolyzed in 25 h by the action of α-glucosidase while no detectable hydrolysis was observed after a 25-h incubation with β-glucosidase. The remaining question is whether the glycosidic linkage involves the C-1 or C-2 of the glycerol moiety. The ^{13}C NMR resonances at 62.3, 61.3, and 61.2 ppm are indicative of three CH_2OH groups, which would occur only if the linkage was 1→2, in agreement with the assignments of Norton et al. (28). Hence, on the basis of enzymatic and chromatographic analysis, ^{13}C NMR spectroscopy, and mass spectrometry, the identity of the major osmolyte in stressed *P. mendocina* SKB70 is *O*-α-D-glucopyranosyl-α-(1→2)-glycerol.

Osmotic regulation of compatible-solute accumulation. Several experiments were carried out to demonstrate that the accumulation of glutamate, NAGGN, and glucosylglycerol is regulated by the osmolarity of the medium. First, cultures were grown at 0.8 M NaCl in two distinct media: MCAA and LAS. ^{13}C NMR spectroscopy revealed that the two cultures accumulated similar levels of the same three osmolytes: glutamate, NAGGN, and glucosylglycerol (Table 1). Hence, the accumulation of these solutes does not depend on the specific carbon and nitrogen sources added to the growth medium.

Also, *P. mendocina* SKB70 was grown in MCAA medium supplemented with NaCl at concentrations ranging from 0 to 1.0 M. Only traces of glutamate were detected by NMR spectroscopy in cells grown in the absence of added NaCl. Significant amounts of NAGGN and glucosylglycerol were not detected until the concentration of salt had reached approximately 0.3 M NaCl (Fig. 2). Then, the intracellular contents of the three solutes increased with salt concentration. Overall, glucosylglycerol was always the most abundant osmolyte, and glutamate and NAGGN were approximately equimolar at all salinity levels (Fig. 2). However, as salinity increased, the heteroside contributed to a lesser fraction of the total pool of cellular osmolytes, representing from 62% of this pool at 0.3 and 0.5 M NaCl to only 37% at 1.0 M NaCl.

Since the data in Fig. 2 indicate that elevated osmolarity stimulates the accumulation of glucosylglycerol, then conversely, an osmotic downshock should cause the intracellular concentration of the osmolyte to decrease. This possibility was

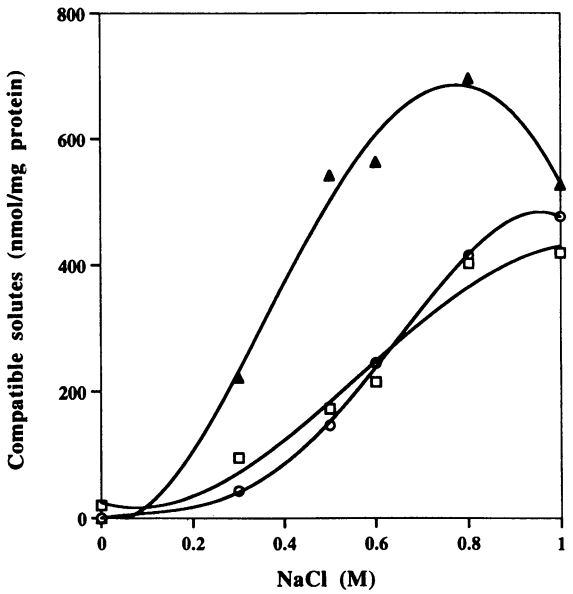

FIG. 2. Intracellular accumulation of compatible solutes in *P. mendocina* SKB70 as a function of NaCl concentration in the medium. Cells were grown in MCAA medium with NaCl as indicated. The amounts of glutamate (□), NAGGN (○), and glucosylglycerol (▲) were quantitated by ^{13}C NMR spectroscopy as described in Materials and Methods.

tested by determining the fate of glucosylglycerol after osmotic downshock. A stressed culture grown at 0.8 M NaCl was shifted to 0.2 M NaCl, and the levels of glucosylglycerol in the cells and in the downshock medium were assayed by HPLC. About 90% of the polyol was recovered in the medium, while about 5% was found in the cellular extract. The remainder may have been lost because of the manipulations of the samples, or it may have been metabolized. The glucosylglycerol found in the medium was not due to cell lysis, since it was found that over 70% of the cells survived this treatment. Hence, the intracellular pool of glucosylglycerol, which is produced in response to hyperosmotic stress, is rapidly depleted by export when the stress is diminished.

Modulation of intracellular osmolyte content by other environmental factors. To determine if the chemical composition of the external osmoticum influences the accumulation of osmolytes in *P. mendocina* SKB70, the osmolarity of the growth medium was increased by the addition of high concentrations of various salts or sucrose, which is metabolically inert in this strain (this study) as it is in most strains of *P. mendocina* (30). Large amounts of glutamate, NAGGN, and glucosylglycerol were found in cells grown in the presence of 0.8 M KCl, 0.7 M K_2SO_4, or 0.7 M Na_2SO_4 (Table 1). Glutamate, NAGGN, and glucosylglycerol were also accumulated in cells grown in 0.6 M sucrose. However, their intracellular levels were considerably lower (40, 50, and 160 nmol/mg of protein, respectively) than in cells grown at high concentrations of NaCl, KCl, K_2SO_4, or Na_2SO_4, and sucrose itself was accumulated as a compatible solute (230 nmol/mg of protein). The lower glutamate, NAGGN, and glucosylglycerol contents are due partly to the lower osmotic pressure of this medium (1.7 MPa) compared with the pressure of the others (approximately 3.6 MPa) and perhaps also to the accumulation of sucrose in the cell, which would reduce the requirement for the other polyol, glucosylglycerol.

Interestingly, the ionic composition of the growth medium also had a significant effect on the quantities of osmolytes in *P. mendocina* SKB70. Indeed, no glucosylglycerol was detected in cells grown in 0.5 M K_2HPO_4; however, a significant level of trehalose was observed. Hence, it appears that under this condition glucosylglycerol was replaced by trehalose, an osmolyte commonly found in bacteria, cyanobacteria, and yeasts (7, 8, 16, 36, 44). Trehalose was also observed in cultures stressed with 0.7 M Na_2SO_4 and 0.7 M K_2SO_4; however, the glucosylglycerol content remained high in cultures stressed with Na_2SO_4 and was reduced by only 30% in cultures stressed with K_2SO_4. Since it has been shown that trehalose accumulation is osmoregulated and/or is growth phase dependent in other bacteria and yeasts (16, 40, 44), cultures of *P. mendocina* SKB70 were also harvested several hours after reaching stationary phase. No trehalose was detected in cells grown for 21 to 43 h in 0.8 M NaCl–MCAA medium. Moreover, in cultures grown with sulfate or phosphate salts, trehalose content did not correlate with the physiological age of the culture (data not shown). Thus, trehalose accumulation may be triggered by high concentrations of sulfate (K_2SO_4 and Na_2SO_4) or phosphate (K_2HPO_4) ions but appears not to be growth phase dependent under our experimental conditions. These results are discussed below.

Role of glycine betaine and proline in osmoregulation of *P. mendocina* SKB70. In a recent review, Csonka (7) reports that both proline and glycine betaine alleviate growth inhibition of an unspecified strain of *P. mendocina* at high osmolarity. The effects of these two compounds on the growth of stressed *P. mendocina* SKB70, therefore, were determined. Cultures were grown in LAS and MCAA media supplemented with 1.0 M NaCl, with or without 1 mM glycine betaine or proline. While proline had no significant beneficial effect on the growth rate nor on the lag phase of the strain in these two media, glycine betaine significantly reduced the lag phase of *P. mendocina* SKB70 from 45 to 20 h in LAS medium and from 20 to 5 h in MCAA medium. Furthermore, glycine betaine slightly improved the growth rate (30 to 40%) of strain SKB70 in these two media, suggesting that glycine betaine participates in osmoadaptation in *P. mendocina* SKB70 whereas proline does not. The apparent discrepancy between our data on osmoprotection of *P. mendocina* by exogenous proline and the report of Csonka (7) could be explained by the utilization of different growth media and/or could be strain related.

The potential role of glycine betaine and proline in osmotic adjustment in *P. mendocina* SKB70 was also investigated by ^{13}C NMR spectroscopy. Cultures were grown in MCAA medium, with or without 0.8 M NaCl and 1 mM glycine betaine or 1 mM proline, and their osmolyte contents were determined. Neither solute was detected in unstressed cells. Also, as anticipated from growth experiments, exogenous proline had no effect on the osmolyte content of salt-stressed *P. mendocina* SKB70 (data not shown). However, exogenous glycine betaine had a profound effect on the osmolyte profile of this strain (Table 2). Indeed, glycine betaine was by far the most abundant osmolyte in cells grown in 0.8 M NaCl plus 1 mM glycine betaine. Moreover, glycine betaine provoked a 3.3-fold decrease in glutamate, a 4.5-fold decrease in glucosylglycerol, and a minor reduction (about 25%) in NAGGN. Hence, glycine betaine acts as an exogenous osmoprotectant, is accumulated intracellularly, and regulates the accumulation of the endogenous osmolytes in *P. mendocina* SKB70. Interestingly, the sum of all organic osmolytes in stressed *P. mendocina* SKB70 grown in the presence of glycine betaine was significantly higher than that in stressed cells grown in its absence (Table 2). These data most likely indicate that the concentrations of organic osmolytes merely compensate for differences in intracellular concentrations of inorganic ions, particularly potassium (43), which we cannot measure by using ^{13}C NMR spectroscopy.

TABLE 2. Effect of glycine betaine added to the growth medium on the osmolyte composition of *P. mendocina* SKB70[a]

Addition to MCAA medium	Amt[b] (nmol/mg of protein) of:			
	Glutamate	NAGGN	GG	GB
None	30	UD	UD	UD
0.8 M NaCl	400	420	700	UD
0.8 M NaCl + 1 mM GB	120	320	160	2,440

[a] To avoid interference with extracellular glycine betaine during the assay, cells were washed in iso-osmotic MCAA medium without glycine betaine prior to extraction. No osmolytes were detectable when 1 mM glycine betaine alone was added to the medium. GG, glucosylglycerol; GB, glycine betaine; UD, undetectable.

[b] Quantitated by ^{13}C NMR spectroscopy as indicated in Materials and Methods.

Osmolyte composition of related *Pseudomonas* species. Prior to this study, stress-related accumulation of glucosylglycerol was documented in cyanobacteria (4, 10, 25, 33), the halophilic phototroph *Rhodobacter sulfidophilus* (38), and the resurrection plant *Myrothamnus flabellifolia* (3) but not in any *Pseudomonas* species or any other nonphotosynthetic bacterium. Thus, it was of interest to determine whether glucosylglycerol is also found in other species in this genus. The osmolyte composition of the type strains of *P. mendocina*, the closely related species *P. pseudoalcaligenes*, and a strain of *P. stutzeri* was determined. The cells were grown in MCAA medium plus 0.8 M NaCl. *P. mendocina* ATCC 25411 and *P. pseudoalcaligenes* ATCC 14440, which were as salt tolerant as *P. mendocina* SKB70 ($[NaCl]_{max}$ = 1.2 M NaCl), also accumulated glutamate, NAGGN, and glucosylglycerol (Table 3), indicating that they all osmoregulate in a similar manner. In contrast, *P. stutzeri* JM300, a more distantly related (14, 30), salt-sensitive species did not accumulate any of these three osmolytes at high osmolarity (data not shown).

DISCUSSION

In this study, the mechanism of osmotic stress adaptation of *P. mendocina* SKB70 was investigated. Natural-abundance ^{13}C NMR spectral analysis showed that stressed cells of *P. mendocina* SKB70 accumulate the polyol *O*-α-D-glucopyranosyl-α-(1→2)-glycerol, the dipeptide NAGGN (39), and L-α-glutamate. The intracellular quantities of these solutes increase with the concentration of NaCl in the growth medium (Fig. 2). Also, the occurrence of these osmolytes in osmotically stressed cells grown in a variety of media unambiguously demonstrates that their accumulation is osmotically regulated and relies on de novo biosynthesis.

TABLE 3. Osmolytes accumulated by *Pseudomonas* strains grown at high osmolarity[a]

Strain	Amt[b] (nmol/mg of protein) of:		
	Glutamate	NAGGN	GG
P. mendocina SKB70	400	420	700
P. mendocina ATCC 25411	410	640	1,080
P. pseudoalcaligenes ATCC 14440	260	250	730

[a] Cultures were grown in MCAA plus 0.8 M NaCl.

[b] Quantitated by ^{13}C NMR spectroscopy as indicated in Materials and Methods. GG, glucosylglycerol.

Hyperosmolarity elicits the biosynthesis and accumulation of glutamate and NAGGN in most *Pseudomonas* species screened thus far, including the type strains of *P. mendocina* and *P. pseudoalcaligenes* (this study), *P. aeruginosa* (9), *P. fluorescens* (39), and *P. putida* (39a). However, NAGGN is not synthesized by the relatively salt-sensitive strain *P. stutzeri* JM300 (this study) or by the extremely salt-tolerant species *P. halosaccharolytica* (which tolerates 3.3 M NaCl) (17) and *P. halophila*, which rely on ectoines as their major endogenous osmolytes (38). NAGGN was also detected as a major osmolyte in all strains of *R. meliloti* screened thus far but was not accumulated in other rhizobia (40). Minor amounts of this dipeptide were also accumulated in slightly to moderately halophilic phototrophic bacteria such as *Rhodopseudomonas marina*, *Chromatium purpuratum*, *Chromatium salexigens*, and *Thiocapsa halophila*, which were isolated from marine salterns (6, 38).

Stress-related accumulation of glucosylglycerol in any *Pseudomonas* species, or a related bacterium, has not been reported previously. In fact, glucosylglycerol was known essentially as the sole organic osmolyte to accumulate in marine cyanobacteria and was therefore considered a key factor for salinity tolerance in these microorganisms (25, 33, 35, 48). Indeed, cyanobacteria unable to synthesize glucosylglycerol following prolonged culture in the dark or mutagenesis exhibit a salt-sensitive phenotype (10, 15). Aside from marine cyanobacteria, glycosylglycerol was also detected as a minor osmolyte in *Rhodobacter sulfidophilus*, a moderately halophilic phototrophic bacterium (38). Several other heterosides structurally analogous to glucosylglycerol also accumulate in salt-stressed eukaryotic red algae (18, 19, 21, 34), in desiccated leaves of the resurrection plant *M. flabellibolia* (3), and in bulbs of several *Lilium* species (3). The accumulation of glucosylglycerol in isolates from two subdivisions of the proteobacteria (i.e., in *R. sulfidophilus* [alpha subclass] [38] and in *P. mendocina* and *P. pseudoalcaligenes* [gamma subclass] [30]), in distantly related cyanobacteria, in red algae, and in higher plants raises two questions. The first is the question of the potential significance of the glucosylglycerol biosynthetic pathway(s) in taxonomy, particularly of the proteobacteria and *Pseudomonas* species. The second is the question of the importance of this pathway(s) in evolution, particularly with respect to the colonization of saline or dry environments. Additional screenings of new isolates representing the broad genetic diversity of these microorganisms may provide an answer to these questions.

The ionic composition of the growth medium had a marked effect on the intracellular concentration of compatible solutes accumulated in stressed *P. mendocina* SKB70. For example, glucosylglycerol was undetectable in cells grown in 0.5 M K_2HPO_4 (Table 1). This observation is not necessarily surprising if the data from the literature are examined. For instance, the activity of isofloridoside-phosphate synthase, a key enzyme of the isofloridoside pathway in the unicellular red alga *Poterioochromonas malhamensis* (19), is strongly inhibited by low concentrations (50 mM) of P_i (20). Moreover, the biosynthesis of galactosylglycerols (floridoside and isofloridoside) in eukaryotic algae involves multiple phosphorylation and dephosphorylation reactions which are tightly regulated and controlled by a variety of environmental factors (19, 22). Considering these data and the probable overlap in the biosynthetic pathways for galactosylglycerols and glucosylglycerol, we speculate that the inability of *P. mendocina* SKB70 to accumulate glucosylglycerol at 0.5 M K_2HPO_4 (Table 1) may be due to the specific inhibition of its biosynthesis by high intracellular phosphate levels. Obviously, further investigations are needed to determine if such phosphate-dependent regulatory mechanisms occur in *P. mendocina* SKB70.

Both high osmolarity and the presence of either phosphate or sulfate ions are required to elicit the accumulation of trehalose (Table 1). This dual modulation is consistent with several other recent reports on the regulation of the intracellular trehalose level by multiple stresses. Some prominent examples include (i) nitrogen limitation and high osmolarity in *Ectothiorhodospira halochloris* (12); (ii) desiccation, heat shock, and carbon starvation in yeasts (44, 46); and (iii) high osmolarity and transition to stationary phase in *Escherichia coli* and *R. meliloti* (16, 40). The absence of trehalose in *P. mendocina* SKB70 grown to late stationary phase in 0.8 M NaCl and the lack of correlation between physiological age and trehalose levels in cultures grown in 0.7 M K_2SO_4, 0.7 M Na_2SO_4, or 0.5 M K_2HPO_4 suggest that the accumulation of trehalose is not developmentally regulated but, rather, may be triggered by high concentrations of sulfate and phosphate ions in the medium. However, further studies are required to establish the basis for this particular ionic effect.

Glycine betaine is accumulated only when it is supplied exogenously to stressed cells of *P. mendocina* (Table 3), indicating that its accumulation cannot occur via de novo biosynthesis. Furthermore, glycine betaine suppressed the accumulation of all three endogenous osmolytes (Table 2). This is a common phenomenon that has been observed in several other bacterial species on which betaine also confers enhanced osmotic tolerance (8, 9, 40, 41, 42). This ability to modulate the accumulation of endogenous osmolytes indicates a clear bacterial preference for glycine betaine which is highly compatible with metabolic functions at high cytoplasmic concentrations (8, 11, 35). It may also illustrate a preference for transport over de novo biosynthesis which is probably bioenergetically more costly and, thus, less conservative for survival under extreme conditions (42). Although glycine betaine is the preferred osmolyte in strain SKB70, it confers only a modest level of enhanced osmotic tolerance compared with other stressed bacterial species that preferentially accumulate betaine. Perhaps the unique combination of NAGGN and glucosylglycerol is almost as osmoprotective (i.e., it stabilizes macromolecular function equally well) as glycine betaine alone. Further investigations are needed to compare the effects of glucosylglycerol, NAGGN, and glycine betaine on the functional stability of macromolecular structures under stress conditions.

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