

## Osmotic adjustment in *Brevibacterium ammoniagenes*: pipecolic acid accumulation at elevated osmolalities

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*Brevibacterium ammoniagenes* ATCC 6872 was grown aerobically in minimal defined glucose media of different osmolalities induced either by NaCl, other electrolytes or non-electrolytes. Growth rate was slightly affected by elevation of medium osmolality up to 1 M-NaCl, but severely decreased at 1.5 M; however even at 2 M-NaCl, slow growth still occurred. Glycine betaine (or its precursor choline) did not stimulate growth in high osmotic media, although it accumulated intracellularly and was not metabolized. An organic solute which increased substantially in concentration during osmotic treatment with various osmolytes, was isolated and identified as pipecolic acid. No accumulation of this imino acid was observed when the medium concentration was raised by adding the permeant glycerol or when the culture medium was supplemented with glycine betaine (1 mM). While this non-proteic cyclic amino acid may play an important role in bacterial adaptation to environmental stress, it did not accumulate to a high level. Preliminary data suggest that the biosynthesis of pipecolic acid from lysine is strongly regulated by external osmolality.

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### Introduction

The ability to adapt, within limits, to changes in the osmotic strength of the environment is a property of most living cells. Many species of bacteria respond to elevated medium osmotic pressure by accumulating inorganic ions and low-molecular-mass organic solutes, such as amino acids and their derivatives, or carbohydrates, to high intracellular concentrations (Brown, 1976; Yancey *et al.*, 1982; Imhoff, 1986). The primary response to osmotic stress in bacteria is the accumulation of K<sup>+</sup> ions (Christian & Waltho, 1961; Epstein, 1986), with a concomitant increase in glutamate concentration (Measures, 1975; Tempest *et al.*, 1970; Botsford, 1984; Hua *et al.*, 1982; Makemson & Hastings, 1979; Yap & Lim, 1983). As a secondary response, the micro-organism can take up large amounts of osmotically active solutes such as proline and betaines. Exogenous osmoprotectants such as proline, choline (converted into glycine betaine intracellularly) and betaines are taken up via osmotically regulated transport systems, stimulating or restoring the growth of stressed cells (Imhoff, 1986; Christian, 1955; Dunlap & Csonka, 1985; Perroud & Le

Rudulier, 1985; Le Rudulier & Bernard, 1986; Bernard *et al.*, 1986).

Most studies on osmoregulation in the bacterial world have been confined to enterobacteria and other Gram-negative bacteria, with only a few reports on Gram-positive bacteria (Hutkins *et al.*, 1987; Whatmore *et al.*, 1990). Despite the fact that coryneform bacteria are among the most useful organisms for the industrial production of amino acids and nucleotides and that the osmotic potential of the medium is an important factor for the production of metabolites, they have been little studied. To our knowledge, osmoprotection has only been investigated in *Brevibacterium lactofermentum* (Kawahara *et al.*, 1989, 1990). This investigation demonstrated the relief of the effects of osmotic stress by endogenous proline and exogenously supplied glycine betaine.

In this paper, we report the ability of *Brevibacterium ammoniagenes* to sustain growth on media of high osmotic strength and the strategy developed by this bacterium to maintain osmotic balance. The effect of the well-known exogenous osmoprotectant glycine betaine was analysed, and a potential new osmoprotectant for bacteria was isolated and subsequently identified as pipecolic acid.

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## Methods

**Culture and growth conditions.** *Brevibacterium ammoniagenes* ATCC 6872 was grown aerobically at 30 °C with constant shaking (130 r.p.m.) on the defined M63 minimal medium (Miller, 1972) using glucose or lactate as carbon source. Minimal media of elevated osmotic strength were obtained by adding NaCl, KCl or the non-electrolytes sucrose, sorbitol or glycerol at the concentrations indicated. The osmoprotectants glycine betaine and choline were added to a concentration of 1 mM. The osmotic pressure of each medium was measured before use by freezing-point determination. An overnight exponential-phase culture on LB medium (Miller, 1972) was used as inoculum at a final concentration of 2% (v/v).

Bacterial growth was monitored as OD<sub>570</sub>. The dry weight (DW) was estimated on washed cells, oven-dried at 80 °C until constant weight. For physiological studies, cells in exponential growth phase were harvested by centrifugation, washed twice with an isotonic solution and maintained at 4 °C until use.

**Extraction of cellular solutes.** The pellet of freshly washed cells was extracted at least twice in 80% (v/v) ethanol under vigorous magnetic stirring at room temperature for 15 min. After centrifugation, the combined supernatants were filtered and evaporated to dryness at 40 °C. The dried extract was dissolved in a minimum volume of distilled water and stored at -20 °C until analysis. This constituted the ethanol-soluble fraction (ESF); the extraction pellet, including the ethanol-insoluble cell components, was termed the EIF fraction.

**Chromatographic analysis.** ESF was used directly or after partial purification by passage through a cation-exchange column (20 cm × 1 cm) of Bio-Rad AG50 × 8, H<sup>+</sup> form. Amino acids were eluted with 2 M-NH<sub>4</sub>OH; the solution was evaporated to dryness and the residue dissolved in 1 ml distilled water. The eluted fraction was subjected to paper chromatography (Whatman 1 and 3MM) or TLC (silica gel 60 F<sub>254</sub>, Merck, 0.2 mm thickness), high-voltage electrophoresis (Whatman 3MM paper, 3% formic acid, electric field value of 40 V cm<sup>-1</sup>), and HPLC.

Paper chromatography and TLC were run in two different solvents: *n*-butanol/acetic acid/water (12:3:5, by vol.) and aqueous phenol (20%, v/v water)/ethanol (1:1, v/v). For amino acid quantification, HPLC analysis was done using the Beckman Gold System equipped with a reversed-phase ultrasphere ODS 5 μm column (4.6 mm × 25 cm). The amino acids were derivatized prior to injection using 4-dimethylaminoazobenzene-4'-sulphonyl chloride (Dabsyl chloride, Fluka) and detected at 436 nm (Chang *et al.*, 1983). An elution gradient starting with a mixture of 12 mM-sodium citrate (83%)/acetonitrile (17%) (both v/v) and ending at 80 min with 100% acetonitrile was programmed. Formamide (4%, v/v) was added to both elution solvents. The flow rate was set at 1.4 ml min<sup>-1</sup> and the column temperature was maintained at 35 °C. 3-Nitro-L-tyrosine (Fluka) was added as the internal standard. All experiments were repeated with less than 5% error.

Proline was detected by paper chromatography and HPLC analysis; alternatively it was assayed as described by Troll & Lindsley (1955).

**Pipecolic acid purification.** Samples for spectral analysis were prepared from cells grown for 36 h in 1 l of M63 medium supplemented with 1 M-NaCl. After centrifugation, the cell pellet was suspended in 80% (v/v) ethanol and extracted three times. Cell debris was removed by centrifugation (3500 g, 10 min) and the resulting ESF was submitted to preparative chromatography on Whatman 3MM paper using aqueous phenol/ethanol (1:1, v/v) as solvent. After drying at 40 °C, the bands corresponding to the unknown compound were localized by spraying with ninhydrin (0.4% in *n*-butanol) while the unsprayed corresponding strips were cut off and eluted with 2 M-HCl. After

evaporation to dryness, the samples were further purified by high-voltage paper electrophoresis (1 h at 30 V cm<sup>-1</sup>), eluted as before and lyophilized. The dry sample (at least 10 mg) was dissolved in D<sub>2</sub>O for <sup>13</sup>C and <sup>1</sup>H NMR analysis using a Bruker AC 300P spectrometer.

**NMR spectroscopy and mass spectrometry.** The natural abundance <sup>13</sup>C NMR spectra were recorded in the pulsed Fourier transform mode at an operational frequency of 75.4 MHz.

The <sup>1</sup>H NMR spectra were recorded at 300 MHz. The solvent (D<sub>2</sub>O) provided the signal for the NMR field lock. Chemical shifts were expressed in parts per million (p.p.m.) downfield from sodium 3-(trimethylsilyl) propane sulphonate (DSS) which was added as an internal reference. The multiplicity of the signals were given as singlets (*s*), doublets (*d*), triplets (*t*), quadruplets (*q*) and multiplets (*m*).

Mass spectrometry was performed using a Varian Mat 311 spectrometer with the following parameters: electron energy, 70 eV; accelerating voltage, 3000 V; temperature of direct-insertion probe, 180 °C.

**Radiolabelling assays.** To determine the effect of salt on lysine uptake and metabolism, L-[U-<sup>14</sup>C]lysine monohydrochloride (11.1 GBq mmol<sup>-1</sup>; Amersham, France) was supplied to a cell suspension grown to mid-exponential phase on minimal medium. Each assay, with or without NaCl, contained, in a total volume of 0.5 ml, labelled lysine at a final concentration of 360 nM (4 × 10<sup>5</sup> d.p.m.). The mixture was incubated with shaking at 30 °C for 2 h and the soluble compounds were extracted with 80% (v/v) ethanol as described above. Respired CO<sub>2</sub> was trapped on a strip of filter paper (1 × 3 cm) moistened with 0.2 ml of 6 M-KOH. Subsamples of ESF, and the whole EIF and CO<sub>2</sub>, were transferred into counting vials with BCS liquid scintillant (Amersham, France). The radioactivity of each fraction was determined using a Packard Tri-Carb spectrometer. A subsample of the insoluble fraction was treated with 6 M-HCl at 105 °C for 20 h in sealed vials and chromatographed in order to determine the nature of labelled compounds incorporated into hydrolysable macromolecular components.

**Transport assays.** [1,2-<sup>14</sup>C]glycine betaine was prepared from [1,2-<sup>14</sup>C]choline (1.6 GBq mmol<sup>-1</sup>) as described by Ikuta *et al.* (1977). [<sup>14</sup>C]choline was enzymically oxidized by choline oxidase from *Alcaligenes* sp. (Sigma). To isolate [<sup>14</sup>C]glycine betaine, the reaction mixture was subjected to high voltage electrophoresis at 40 V cm<sup>-1</sup>, with Whatman 3MM paper previously moistened in 3% (v/v) formic acid (pH 2.0). The [<sup>14</sup>C]glycine betaine was thoroughly eluted with 10 mM-HCl which was then removed by rotary evaporation. The purity was checked by co-electrophoresis and co-chromatography with pure glycine betaine.

Cells used for transport assays were grown to an OD<sub>570</sub> of 0.5, harvested by centrifugation (3500 g for 10 min), washed twice with growth medium lacking carbon source and concentrated to OD<sub>570</sub> = 1 in the same solution. Transport was initiated by adding 1 ml of cell suspension to 25 μl [<sup>14</sup>C]glycine betaine or [<sup>14</sup>C]choline (20 × 10<sup>3</sup> d.p.m., 0.16 μM). Samples (0.1–0.2 ml) were taken at intervals and rapidly collected on 2.5 cm glass-fibre filters (Whatman type GF/F) and washed twice with a solution of the suspension medium. Dried filters were introduced into scintillation vials containing 6 ml BCS. Radioactivities were determined in a Packard Tri-Carb spectrometer. In all experiments, the total concentration of glycine betaine or choline in the transport assays was adjusted so that no more than 20% of the substrate was taken up during the course of the experiment. When chloramphenicol was used, the inhibition of growth was verified by monitoring cell density, which did not increase within 1 h after addition of the antibiotic. All data presented are mean values of duplicates from at least three different experiments, and results agreed within less than 10% deviation.

## Results

### Effect of increased osmolality on bacterial growth

To analyse the response to increased medium osmolality, *Brevibacterium ammoniagenes* was grown on minimal M63 medium with NaCl at different concentrations from 0–1.5 M. The osmotic pressures developed by these culture media were [osmol (kg water)<sup>-1</sup>]: 0.26 (no added NaCl); 1.12 (0.5 M-NaCl); 2.20 (1.0 M-NaCl); and 2.96 (1.5 M-NaCl).

Exposure to elevated osmotic strength reduced the growth rate from 0.20 generations h<sup>-1</sup> in minimal medium without added NaCl, to 0.14 generations h<sup>-1</sup> in 1 M-NaCl (Fig. 1). At salinities above 1 M-NaCl, growth rate decreased sharply with only 0.04 generations h<sup>-1</sup> in 1.5 M-NaCl. At 2 M-NaCl, only very slow growth occurred (data not shown). The culture absorbance at the stationary phase decreased from 0 to 0.5 M-NaCl, but no significant change appeared from 0.5 to 1 M. In 1.5 M NaCl, the absorbance reached only 10% of that obtained with cells grown without NaCl.

Due to probable changes in cell size during osmolality enhancement, leading to modification in light scattering and hence in absorbance (Koch, 1984), it seems preferable to define growth in terms of cell biomass. Dry weight was measured from bacterial suspensions grown at different salinities (0, 0.5, 1.0 and 1.5 M-NaCl) and harvested at the termination of growth. Cell biomass showed a 57% decrease at 0.5 M-NaCl, 68% at 1 M and 93% at 1.5 M, compared to controls grown in the absence of NaCl.

When the electrolyte KCl, or the non electrolytes sucrose or sorbitol, were substituted for NaCl as osmotic agents, at similar osmolalities, a comparable reduction in growth parameters was observed. In contrast, replacing NaCl with glycerol, which freely permeates the cells, did not affect bacterial growth (data not shown).

### Role of glycine betaine and choline in the osmoregulation of *B. ammoniagenes*

We examined the effects of glycine betaine and choline on the growth of *B. ammoniagenes* in minimal medium M63 with various types of osmotic solutes. The addition of glycine betaine or choline to media of high osmotic strength did not show any effect on either growth rate or yield (Fig. 1). This could have resulted from the absence of uptake systems for these osmoprotectants in *B. ammoniagenes*. We therefore investigated the transport characteristics of these compounds. Glycine betaine uptake was determined in cells grown in M63 medium with no added NaCl or 1 M-NaCl. The rate of betaine uptake was directly correlated with the medium osmola-

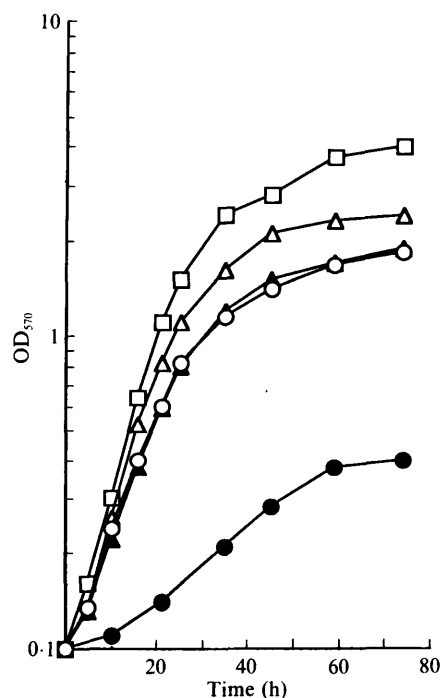


Fig. 1. Effect of NaCl concentration on growth of *B. ammoniagenes*. Cells were grown aerobically at 30 °C in M63 medium. Symbols: □, no added NaCl; △, 0.5 M-NaCl; ▲, 1 M-NaCl; ●, 1.5 M-NaCl; ○, 1 M-NaCl with 1 mM-glycine betaine.

lity, and increased from 1.5 to 9 nmol min<sup>-1</sup> (mg dry weight)<sup>-1</sup>. Increasing medium osmolality to the same extent by NaCl, KCl or sucrose resulted in a similar stimulation of betaine uptake, indicating that this stimulation was a consequence of increased osmolality rather than of increased salinity.

When cells were grown on media lacking NaCl, and upshocked with 1 M-NaCl medium, we observed an immediate stimulation of betaine transport in non-growing cells; the transport activity was identical to that of strains grown in 1 M-NaCl medium. If the cells were treated with chloramphenicol prior to upshock, no effect of the protein synthesis inhibitor was detected on betaine transport elicited by osmotic stress. From these data, we conclude that the betaine transport system is not induced by high osmotic pressure but is stimulated by elevated extracellular osmotic pressure.

The uptake of choline was also examined in cells grown with no added NaCl or 1 M-NaCl media. Choline uptake rate was very slow for cells grown at low osmolality and was only slightly increased when cells were grown in 1 M-NaCl medium.

We investigated whether *B. ammoniagenes* degraded choline and glycine betaine by cultivation in minimal medium with choline or glycine betaine as sole carbon or

nitrogen source and 0, 0.2, and 0.5 M-NaCl. No growth of *B. ammoniagenes* was observed after 4 d of incubation.

A radioactive labelling experiment was carried out to investigate the metabolic fate of these compounds. *B. ammoniagenes* was grown for 12 h with [1,2-<sup>14</sup>C]choline or glycine betaine in M63, in the absence and presence of 1 M-NaCl. Glucose was used as carbon source. The cells were then extracted with alcohol to release their soluble components. For glycine betaine, 94 and 96% of the incorporated radioactivity was recovered in the soluble fraction for cells grown with 0 and 1 M-NaCl, respectively. When the soluble fractions were analysed by chromatographic and electrophoretic techniques, all the radiocarbon was associated with glycine betaine.

When [<sup>14</sup>C]choline was incorporated into the medium, only a small fraction of the supplied radiocarbon was taken up by the cell at low osmolality, while up to 95% was absorbed at high osmolality. The soluble fraction contained most of the incorporated radiolabel; 97 and 91%, for cells grown at low and high osmolality, respectively. After chromatographic and electrophoretic analysis of the soluble fraction, all the radioactivity was again localized in the glycine betaine spot.

To investigate the possibility of catabolic inhibition of choline and glycine degradation by glucose, the same experiments were repeated using lactate as carbon source. No difference was observed in the results; in all cases, labelled molecules were incorporated intracellularly and recovered in the soluble fraction as glycine betaine. We conclude that *B. ammoniagenes* could take up glycine betaine from the growth medium. Choline was transported slowly and readily transformed into glycine betaine. *B. ammoniagenes* is therefore similar to many species of bacteria which possess a choline oxidase but are unable to catabolize glycine betaine.

#### Identification of endogenous accumulated solutes

Since an appreciable growth rate was still observed at quite high osmolality, one might expect the synthesis and accumulation of other endogenous solute(s) to balance the high external osmotic pressure. Chromatographic and electrophoretic analyses were undertaken from ethanol-soluble fractions of cells grown on low or elevated osmotic strength media. Neither proline nor onium compounds, generally associated with osmotic stress, could be detected on chromatograms sprayed either with ninhydrin or Dragendorff reagents. However one spot appeared constantly after ninhydrin spraying and was highly dependent on the level of salt addition. Its  $R_F$  value on chromatograms developed with *n*-butanol/acetic acid/water (12:3:5, by vol.) was 0.52, and in the aqueous phenol/ethanol system (1:1, v/v), was 0.75. The distance of migration in an electric field (30 V cm<sup>-1</sup>),

relative to that of choline, was  $R_c = 0.39$ . The corresponding spot gave a blue-purple colour with ninhydrin reagent, which exhibited a red fluorescence when exposed to 280 nm UV light. The spot size and coloration intensity was directly correlated with salt concentration in the culture media up to 1 M. Above this concentration, no reproducible data were obtained.

The corresponding solute, suspected to be an amino acid due to its behaviour in an electric field and its reaction with ninhydrin, was isolated by preparative paper chromatography with phenol/ethanol as solvent and subjected to further purification using paper electrophoresis. <sup>13</sup>C and <sup>1</sup>H NMR spectroscopic and mass spectrometric analyses were carried out for structural determination.

The following chemical shifts were recorded for <sup>1</sup>H (300 MHz,  $\delta$  p.p.m./DSS): 3.57 (*m*, 1H); 3.35 (*m*, 1H); 2.93 (*t*, 1H); 2.16 (*m*, 1H); 1.79 (*m*, 2H); 1.55 (*m*, 3H) and for <sup>13</sup>C (74.5 MHz): 23.9 (C<sub>4</sub>, C<sub>5</sub>); 28.2 (C<sub>3</sub>); 46.5 (C<sub>6</sub>); 59.2 (C<sub>2</sub>); 174.1 (C=O). All these data were identical to those obtained from commercially available pipecolic acid.

In the mass spectrum, the molecular peak M<sup>+</sup> was at  $m/z = 129$ , corresponding to C<sub>6</sub>H<sub>11</sub>NO<sub>2</sub>; the base peak M<sup>+</sup>-CO<sub>2</sub>H = 84, was significant; calculated mass = 129.078; found = 129.079.

These data indicated the isolated compound to be pipecolic acid. Comparative co-chromatography with an authentic sample confirmed our predictions.

#### Effect of osmotic strength on the accumulation of pipecolic acid

Pipecolic acid content was determined using an HPLC technique. Data presented in Table 1 show that the content increased with medium osmolality from 40.3 in a two-fold diluted M63 medium to 130.8 nmol (g dry weight)<sup>-1</sup> in a medium containing 1 M-NaCl. The addition of 1 mM-glycine betaine to the culture medium did not affect the content of pipecolic acid in the absence

Table 1. Effect of increasing osmolality on the accumulation of intracellular pipecolic acid in *B. ammoniagenes*.

Values are means of duplicate determinations

Media [NaCl]	Pipecolic acid [nmol (mg dry weight) <sup>-1</sup> ]
diluted 0 M*	40.3
0 M	42.4
0.5 M	75.3
0.8 M	109.2
1.0 M	130.8
0 M + glycine betaine	43.4
1 M + glycine betaine	32.0

\* Minimal medium without added NaCl, diluted twice with H<sub>2</sub>O.

Table 2. Incorporation of  $^{14}\text{C}$  from lysine into pipecolic acid and the insoluble fraction (EIF)

Results are expressed as percent of total incorporated [ $^{14}\text{C}$ ]lysine. The 100% value is  $10.6 \text{ MBq (g dry weight)}^{-1}$  which remained constant independent of medium osmolality; non-metabolized  $^{14}\text{C}$ -lysine and minor labelled compounds constitute the rest of the radioactivity. Values are means of duplicate determinations.

Media [NaCl]	Pipecolic acid (%)	EIF (%)
0 M	28.3	41.2
0.5 M	52.9	23.1
1.0 M	64.6	14.2
1.5 M	72.8	4.2
2.0 M	51.8	2.0
1.0 M + glycine betaine	43.9	31.3

of NaCl, but lowered the content to 32 nmol (mg DW) $^{-1}$  in 1 M-NaCl medium (Table 1).

In order to determine whether pipecolic acid accumulation was the result of salt or osmotic stress, we determined the quantities accumulated when added NaCl (0.5 and 1 M) was replaced by other solutes at identical osmolalities: KCl (0.5 and 1 M), sucrose (0.6 and 1.1 M), sorbitol (0.92 and 1.8 M) and glycerol (0.8 and 1.5 M). KCl, sucrose and sorbitol exerted approximately the same effect as NaCl, whereas glycerol had no effect on intracellular pipecolic acid content.

#### Effect of increased osmolalities on $^{14}\text{C}$ -pipecolic acid biosynthesis

Lysine is generally considered the main precursor of pipecolic acid (Stewart & Larher, 1980). Cells of *B. ammoniagenes* fed with  $^{14}\text{C}$ -lysine incorporated radioactivity at a rate independent of medium osmolality. Both soluble (ESF) and insoluble (EIF) fractions were labelled. Moreover, substantial radioactivity was measured in carbon dioxide, mainly from the cells grown under the lowest salt concentration. Data given in Table 2 show that the labelling of the insoluble fraction (due to lysine incorporation into proteins) decreases sharply from 41.2% of the total radioactivity in the absence of NaCl to only 2% at 2 M-NaCl. In contrast, the radioactivity in the corresponding soluble fraction increased considerably to 98.4% of the supplied  $^{14}\text{C}$ . Only two major radioactive compounds were detected in the soluble fraction: the non-metabolized lysine and pipecolic acid. One of the minor unidentified labelled compounds could correspond to  $\alpha$ -amino adipic acid, a product of lysine oxidation reported in animal cells (Vianey-Liaud *et al.*, 1991; Mihalik *et al.*, 1991). Pipecolic acid accounted for the majority of the metabolized  $^{14}\text{C}$ -lysine (more than 70% of the total at

1.5 M-NaCl). No labelled cadaverine, the decarboxylation product of lysine, was detected on chromatograms.

When glycine betaine was added to the reaction mixture, incorporation of  $^{14}\text{C}$  into pipecolic acid was markedly inhibited while incorporation into protein was stimulated (Table 2).

## Discussion

This study presents evidence that *B. ammoniagenes*, when exposed to increasing medium osmolality, is capable of growth at relatively high external osmotic pressures and hence is able to perform intracellular osmotic adjustment. In *B. ammoniagenes*, glycine betaine transport and its accumulation were stimulated in media of high osmolality. This accumulation may result from the direct uptake of glycine betaine or from its synthesis from choline. In *B. ammoniagenes*, unlike *B. lactofermentum* (Kawahara *et al.*, 1990) and most non-halophilic bacteria (Csonka, 1989), glycine betaine apparently does not stimulate growth rate in media of inhibitory osmotic strength. The absence of any apparent osmoprotective effect of choline and glycine betaine has also been described for another corynebacterium, *Arthrobacter pascens* (Rozwadowski *et al.*, 1991). In this case, choline and glycine betaine appear to have no osmolyte function but are used as carbon sources. This is not the case in *B. ammoniagenes*, since glycine betaine is not metabolized either in low- or high-osmotic-strength media. Glycine betaine uptake activity appears to be regulated by activation of the betaine carrier; such results have been already reported for betaine uptake in another Gram-positive bacterium (*Lactobacillus acidophilus*) by Hutkins *et al.* (1987) and is part of the betaine uptake system in enterobacteria (Csonka, 1989). By analogy to the models proposed for  $\text{K}^+$  and carbohydrate transport systems in *E. coli* (Epstein, 1986; Roth *et al.*, 1985) the deformation of cell membranes caused by a change in turgor alters the conformation of the membrane embedded carrier proteins, stimulating the inward transport of betaine.

*B. lactofermentum* grown in high osmotic strength media accumulates proline. Glycine betaine added to the growth media is taken up rapidly and accumulated into the cell in preference to proline (Kawahara *et al.*, 1990). A similar phenomenon occurs in *B. ammoniagenes*; in this bacterium, glycine betaine tends to repress pipecolic acid synthesis, and perhaps accumulation of other unidentified compounds. Thus, in spite of its apparent inefficiency on growth stimulation, glycine betaine must be considered an osmoprotective compound for *B. ammoniagenes*.

The non-proteic pipecolic acid accumulated as a response to osmotic constraint in *B. ammoniagenes*

(Table 1). It is the first time that this phenomenon has been reported in bacteria, as far as we are aware. Intracellular pipecolic acid content was closely related to elevation of external osmotic pressure, independent of the solute used as long as this solute was not penetrant. The permeant solute glycerol did not trigger any imino acid accumulation; this may indicate that loss of cell turgor, rather than increasing medium osmolality, was the mechanism leading to solute accumulation. Pipecolic acid did not accumulate when the culture medium was supplemented with glycine betaine, suggesting that this potent osmoprotectant could efficiently replace the endogenously accumulated imino acid. Similar results had been reported for other micro-organisms, and it is generally accepted that stressed cells prefer to take up osmoprotectant rather than synthesize endogenous osmolyte, which is energetically expensive (Whatmore *et al.*, 1990; Herzog *et al.*, 1990; Strøm *et al.*, 1986).

With the exception of several reports on lysine metabolism in *Pseudomonas putida* (Basso *et al.*, 1962; Miller & Rodwell, 1971; Perfetti *et al.*, 1972) little is known concerning the occurrence and metabolism of pipecolic acid in bacteria. In plants, a few studies have reported the occurrence of pipecolic acid together with proline in response to osmotic stress (Goas *et al.*, 1976; Stewart & Larher, 1980), but it is unclear why bacteria should accumulate this unusual solute. Should it be considered as a stress signal or rather as a genuine osmoprotectant? Indeed, the maximum pipecolic acid content [130 nmol (g DW)<sup>-1</sup>] at a salinity of 1 M is markedly lower than values reported from other organisms at similar salinities, e.g. 260 nmol of the cyclic amino acid ectoine in *Halomonas elongata* (Wohlfarth *et al.*, 1990) or 300 nmol of proline in *B. lactofermentum* (Kawahara *et al.*, 1989). Moreover since the natural abundance <sup>13</sup>C NMR analysis of a raw ethanol extract does not exhibit any significant signal for pipecolic acid (data not shown), this imino acid might not be considered to be the major osmoprotectant in *B. ammoniagenes*.

The accumulation of pipecolic acid is a further example of the remarkable similarities between bacteria and plants in their response to osmotic stress, and suggests that there may be close parallels in the mechanisms they employ to relieve it.

Experiments using <sup>14</sup>C-lysine show that this amino acid is the probable precursor of pipecolic acid in *B. ammoniagenes*. The rate of this metabolic step was osmolality-dependent and severely inhibited in the presence of exogenously-supplied osmoprotectant betaine. At low osmolality, pipecolic acid can be metabolized, giving  $\alpha$ -amino adipic acid.

The data presented in this paper imply that pipecolic acid may play an osmotic role in *B. ammoniagenes*, but

further investigations are now needed to establish the metabolic pathways and the mechanisms involved in the regulation of pipecolic acid synthesis.

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## References

- BASSO, L. V., RAO, D. R. & RODWELL, V. W. (1962). Metabolism of pipecolic acid in a *Pseudomonas* species. II  $\Delta^1$ -piperidine-6-carboxylic acid and  $\alpha$ -amino adipic acid- $\delta$ -semialdehyde. *Journal of Biological Chemistry* **237**, 2239–2245.
- BERNARD, T., POCARD, J. A., PERRAUD, B. & LE RUDULIER D. (1986). Variations in the response of salt-stressed *Rhizobium* strains to betaines. *Archives of Microbiology* **143**, 359–364.
- BOTSFORD, J. L. (1984). Osmoregulation in *Rhizobium meliloti*: inhibition of growth by salts. *Archives of Microbiology* **137**, 124–127.
- BROWN, A. D. (1976). Microbial water stress. *Bacteriological Reviews* **40**, 803–846.
- CHANG, Y. Y., KNETCH, R. & BRAUN, D. G. (1983). Amino acid analysis in the picomole range by precolumn derivatization and high performance liquid chromatography. *Methods in Enzymology* **91**, 41–48.
- CHRISTIAN, J. H. B. (1955). The influence of nutrition on the water relations of *Salmonella oranienburg*. *Australian Journal of Biological Science* **8**, 75–82.
- CHRISTIAN, J. H. B. & WALTHO, J. (1961). The sodium and potassium content of non-halophilic bacteria in relation to salt tolerance. *Journal of General Microbiology* **25**, 97–102.
- CSONKA, L. N. (1989). Physiological and genetic responses of bacteria to osmotic stress. *Microbiological Reviews* **53**, 121–147.
- DUNLAP, V. J. & CSONKA, L. N. (1985). Osmotic regulation of L-proline transport in *Salmonella typhimurium*. *Journal of Bacteriology* **163**, 296–304.
- EPSTEIN, W. (1986). Osmoregulation by potassium transport in *Escherichia coli*. *FEMS Microbiology Letters* **39**, 73–78.
- GOAS, M., GOAS, G. & LARHER, F. (1976). Formation de l'acide pipécolique chez *Triglochin maritima*. *Canadian Journal of Botany* **54**, 1221–1227.
- HERZOG, M., GALINSKI, E. A. & TRÜPER, H. G. (1990). Degradation of the compatible solute trehalose in *Ectothiorhodospira halochloris*: isolation and characterization of trehalase. *Archives of Microbiology* **153**, 600–606.
- HUA, S. S. T., TSAI, V. Y., LICHENS, G. M. & NOMA, A. T. (1982). Accumulation of amino acids in *Rhizobium* sp. strain WR 1001 in response to sodium chloride salinity. *Applied and Environmental Microbiology* **44**, 135–140.
- HUTKINS, R. W., ELLEFSON, W. L. & KASHKET, E. R. (1987). Betaine transport imparts osmotolerance on a strain of *Lactobacillus acidophilus*. *Applied and Environmental Microbiology* **53**, 2275–2281.
- IKUTA, S., MATUURA, K., IMAMURA, S., MISAKI, H. & HORIUTI, Y. (1977). Oxidative pathway of choline to betaine in the soluble fraction prepared from *Arthrobacter globiformis*. *Journal of Biochemistry* **82**, 157–163.
- IMHOFF, J. F. (1986). Osmoregulation and compatible solutes in eubacteria. *FEMS Microbiology Reviews* **39**, 57–66.
- KAWAHARA, Y., OHSUMI, T., YOSHIHARA, Y. & IKEDA, S. (1989). Proline in the osmoregulation of *Brevibacterium lactofermentum*. *Agricultural and Biological Chemistry* **53**, 2475–2479.
- KAWAHARA, Y., YOSHIHARA, Y., IKEDA, S. & HIROSE, Y. (1990). Effect of glycine betaine, an osmoprotective compound on the growth of *Brevibacterium lactofermentum*. *Applied Microbiology and Biotechnology* **33**, 574–577.
- KOCH, A. L. (1984). Shrinkage of growing *Escherichia coli* cells by osmotic stress. *Journal of Bacteriology* **159**, 919–924.

- LE RUDULIER, D. & BERNARD, T. (1986). Salt tolerance in *Rhizobium*: a possible role for betaines. *FEMS Microbiology Reviews* **39**, 67–72.
- MAKEMSON, J. C. & HASTINGS, J. W. (1979). Glutamate functions in osmoregulation in a marine bacterium. *Applied and Environmental Microbiology* **38**, 178–180.
- MEASURES, J. C. (1975). Role of amino acids in osmoregulation of non-halophilic bacteria. *Nature, London* **257**, 398–400.
- MIHALIK, S. J., MCGUINNESS, M. & WATKINS, P. A. (1991). Purification and characterization of peroxisomal L-pipecolic acid oxidase from monkey liver. *Journal of Biological Chemistry* **266**, 4822–4830.
- MILLER, D. L. & RODWELL, V. W. (1971). Metabolism of basic amino acids by *Pseudomonas putida*. Properties of the inducible lysine transport system. *Journal of Biological Chemistry* **246**, 1765–1771.
- MILLER, J. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- PERFETTI, R., CAMPBELL, R., TITUS, J. & HARTLINE, R. A. (1972). Catabolism of pipecolate to glutamate in *Pseudomonas putida*. *Journal of Biological Chemistry* **247**, 4089–4095.
- PERRAUD, B. & LE RUDULIER, D. (1985). Glycine betaine transport in *Escherichia coli*: osmotic modulation. *Journal of Bacteriology* **161**, 393–401.
- ROTH, W. G., PORTER, S. E., LECKIE, M. P., PORTER, B. E. & DIETZLER, D. N. (1985). Restoration of cell volume and the reversal of carbohydrate transport and growth inhibition of osmotically upshocked *Escherichia coli*. *Biochemical and Biophysical Research Communications* **126**, 442–449.
- ROZWADOWSKI, K. L., KHACHATOURIANS, G. G. & SELVARA, J. G. (1991). Choline oxidase, a catabolic enzyme in *Arthrobacter pascens*, facilitates adaptation to osmotic stress in *Escherichia coli*. *Journal of Bacteriology* **173**, 472–478.
- STEWART, G. R. & LARHER, F. (1980). Accumulation of amino acids and related compounds in relation to environmental stress. *The Biochemistry of Plants* **5**, 609–635.
- STRØM, A. R., FALKENBERG, P. & LANDFALD, B. (1986). Genetics of osmoregulation in *Escherichia coli*: uptake and biosynthesis of organic osmolytes. *FEMS Microbiology Reviews* **39**, 79–86.
- TEMPEST, D. W., MEERS, J. L. & BROWN, C. M. (1970). Influence of environment on the content and composition of microbial free amino acid pools. *Journal of General Microbiology* **64**, 171–185.
- TROLL, W. & LINDSLEY, J. (1955). A photometric method for the determination of proline. *Journal of Biological Chemistry* **215**, 655–660.
- VIANEY-LIAUD, C., DIVRY, P., POINAS, C. & MATHIEU, M. (1991). Le métabolisme de la lysine chez l'homme. *Annales de Biologie Clinique* **49**, 18–26.
- WHATMORE, A. M., CHUDEK, J. A. & REED, R. H. (1990). The effects of osmotic upshock on the intracellular solute pools of *Bacillus subtilis*. *Journal of General Microbiology* **136**, 2527–2535.
- WOHLFARTH, A., SEVERIN, J. & GALINSKI, E. A. (1990). The spectrum of compatible solutes in heterotrophic halophilic eubacteria of the family Halomonadaceae. *Journal of General Microbiology* **136**, 705–712.
- YANCEY, P. H., CLARK, M. E., HAND, S. C., BOWLUS, R. D. & SOMERO, G. N. (1982). Living with water stress: evolution of osmolyte systems. *Science* **217**, 1214–1222.
- YAP, S. F. & LIM, S. T. (1983). Response of *Rhizobium* sp. UMKL 20 to sodium chloride stress. *Archives of Microbiology* **135**, 224–228.