Succinate-mediated catabolite repression control on the production of glycine betaine catabolic enzymes in *Pseudomonas aeruginosa* PAO1 under low and elevated salinities

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Glycine betaine (GB) and its immediate precursors choline and carnitine, dimethylsulfonioacetate, dimethylsulfoniopropionate, ectoine and proline were effective osmoprotectants for *Pseudomonas aeruginosa*, but pipecolate, trehalose and sucrose had no osmoprotective effect. GB was accumulated stably or transiently when succinate or glucose, respectively, was used as a carbon and energy source. The catabolite repression mediated by succinate occurred at both low and high salinities, and it did not involve the global regulators Vfr and Crc. A proteomic analysis showed that at least 21 proteins were induced when GB was used as a carbon and energy source, and provided evidence that succinate repressed the synthesis of all these proteins. Many of the proteins induced by GB (sarcosine oxidase, serine hydroxymethyltransferase and serine dehydratase) are involved in GB catabolism. In addition, GB uptake was stimulated at high medium osmolalities but it was insensitive to catabolite repression by succinate. Despite its ability to inhibit betaine catabolism, succinate did not allow any better growth of *P. aeruginosa* cells under hyperosmotic constraint. Conversely, as observed for cells supplied with glucose, a transient accumulation of GB was sufficient to provide a significant cell osmoprotection.

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INTRODUCTION

The ability of most micro-organisms to thrive under frequently highly stressful circumstances depends on the sensing of environmental changes and responding to these challenges via highly integrated stress reactions (Bremer & Krämer, 2000). One critical environmental parameter is the osmolality or the salinity of the extracellular medium. Both soil and saline habitats undergo frequent changes in this physico-chemical parameter because rainfall and evaporation cause drastic changes in the environmental osmolality. Despite variations in the osmotic pressure of their environment, bacteria maintain their cytoplasmic hydration level. This may be accomplished by accumulating solutes when extracellular osmolality rises and by rapidly releasing those solutes when extracellular osmolality declines (Wood *et al.*, 2001). The vast majority of prokaryotes cope with increasing osmolality by uptake or synthesis of organic molecules;

among these, those most compatible with cell physiology appear to be preferred (Wood *et al.*, 2001). The spectrum of compatible solutes used by organisms from the three domains of life comprises only a limited number of compounds, which can be divided into two major groups: (i) sugars and polyols, and (ii) α - and β -amino acids and their derivatives, including methylamines (Roeßler & Müller, 2001). The preferred compatible solute for the majority of prokaryotes, and perhaps for the most widely utilized osmolyte, spanning both the plant and animal kingdoms, is the trimethylammonium compound glycine betaine (GB) (Bremer & Krämer, 2000; Robert *et al.*, 2000; Sleator & Hill, 2002; Talibart *et al.*, 1997).

Pseudomonas aeruginosa, a versatile Gram-negative bacterium, can grow in soil, marshes and coastal marine habitats, as well as on plants and in animal tissues; it is an opportunistic pathogen that primarily affects cystic fibrosis patients and immuno-compromised patients. The investigation of osmoadaptive mechanisms in *P. aeruginosa* PAO1 showed that osmotically stressed cells synthesized and accumulated glutamate, trehalose and *N*-acetylglutaminylglutamine amide (NAGGN) in the absence of exogenously provided

Abbreviations: CRC, catabolite repression control; 2D, two-dimensional; 2DE, two-dimensional electrophoresis; GB, glycine betaine; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; NAGGN, *N*-acetylglutaminylglutamine amide; PLC, phospholipase C.

osmoprotectants, while they preferentially accumulated GB when this compound was available in the growth medium. Moreover, the accumulation of exogenous GB led to a dramatic reduction in the intracellular levels of the three endogenous osmolytes (D'Souza-Ault *et al.*, 1993). GB and its precursors choline and carnitine serve as osmoprotectants, but only GB is accumulated as a cytosolic osmolyte under hyperosmolar conditions by *P. aeruginosa* (Lucchesi *et al.*, 1995). In addition to their important role in osmoprotection, choline, carnitine and GB can also support the growth of *P. aeruginosa* at low osmolality, serving as both carbon and nitrogen sources (Lucchesi *et al.*, 1995; Serra *et al.*, 2002). A few reports have proposed a pathway for GB catabolism in bacteria such as *Sinorhizobium meliloti* (Smith *et al.*, 1988) and *Arthrobacter* spp. (Meskys *et al.*, 2001). Under aerobic conditions, bacterial degradation of GB often proceeds by three successive demethylations through the formation of dimethylglycine and sarcosine as intermediates; the resulting glycine is, in turn, converted to serine (Meskys *et al.*, 2001; Smith *et al.*, 1988). In *P. aeruginosa*, one important step towards the genetic and biochemical elucidation of the GB catabolic route was the identification of the GB transmethylase (Gbt), which catalyses the first demethylating step of GB (Serra *et al.*, 2002). Many reports on *P. aeruginosa* showed a link between osmotic stress responses and the synthesis of virulence factors, such as alginate (Berry *et al.*, 1989; Deretic *et al.*, 1990) and phospholipase C (PLC) (Shortridge *et al.*, 1992). GB and its precursors choline and carnitine induce the synthesis of PLC (Lucchesi *et al.*, 1995); moreover, this osmoprotectant-dependent expression of PLC is subject to catabolite repression control (CRC) by succinate, but the global regulators Crc and Vfr are not involved in this mechanism (Sage & Vasil, 1997). Also, an active transport system induced by choline is repressed by succinate, the preferred source of carbon in *P. aeruginosa* (Salvano *et al.*, 1989).

In this paper, we show by a proteomic approach that 21 proteins were induced by GB and most of them were involved in GB degradation. The synthesis of all these proteins was not induced in the presence of a mixture of GB and succinate, but occurred for most of them when glucose was supplied with GB. This is corroborated by the fact that GB, while improving growth at inhibitory osmolality, was accumulated stably in succinate-grown cells, while it was accumulated only transiently in glucose-grown cells. These data suggest that GB utilization in *P. aeruginosa* is controlled by a succinate CRC mechanism at both low and high osmolalities.

METHODS

Strains, media and growth conditions. The following strains were used in this study: *P. aeruginosa* PAO1 (Holloway *et al.*, 1979) and its derivatives PAO8020 (PAO1 Δ crc; Tc^r) (MacGregor *et al.*, 1996), PAO9001 [PAO1 Δ (*orfX*, *vfr*, *trpC*) *aacCI*; Gm^r] (Suh *et al.*, 2002) and PAO9002 (PAO1 *vfr101::aacCI*; Gm^r) (Suh *et al.*, 2002). These strains were grown aerobically at 37 °C with constant shaking (175 r.p.m.) in LB and defined M63 media (Miller, 1972). Glucose

(10 mM), succinate (20 mM) and GB (10 mM) were added to M63 media as the carbon source. The osmotic strength of the media was increased by the addition of NaCl or sucrose as necessary. The antibiotics tetracycline and gentamicin were used in solid media at final concentrations of 50 µg ml⁻¹. Bacterial growth was monitored by OD₅₇₀ measurements.

Extraction and analysis of cellular solutes. The pellets of freshly harvested and washed cells were extracted in 80% (v/v) ethanol with stirring at room temperature for 30 min. After centrifugation, the supernatant (ethanol-soluble fraction) was evaporated to dryness at 40 °C, dissolved into distilled water and stored at -20 °C until needed for further determination. This fraction was analysed by paper chromatography, TLC and paper high-voltage electrophoresis as described by Bernard *et al.* (1993). For spectroscopic analysis, the fraction was evaporated to dryness, and the dry residue was dissolved into 1 ml D₂O; the ¹³C NMR spectrum was recorded as described previously (Bernard *et al.*, 1993; Jebbar *et al.*, 1997).

Radiolabelling assays. Transport and metabolic assays were done with [methyl-¹⁴C]GB (2.07 GBq mmol⁻¹), which was prepared from [methyl-¹⁴C]choline chloride (2.07 GBq mmol⁻¹) as described previously (Bernard *et al.*, 1993; Jebbar *et al.*, 1992). Transport assays were carried out as described by Choquet *et al.* (2005); the GB concentration in the uptake assay was 10 µM. To study the fate of GB or choline, cells were cultured in M63 with or without 0.5 M NaCl, and supplemented with glucose (10 mM) or succinate (20 mM) as the carbon source, and 1 mM [¹⁴C]GB (5.5 MBq mmol⁻¹) or 1 mM [¹⁴C]choline (5.5 MBq mmol⁻¹). After two to five generations, 1 ml cell culture was harvested by centrifugation (5000 g for 10 min) and washed twice with carbon-free M63 containing the same NaCl or sucrose concentration as the growth medium. After extraction in 80% ethanol, the radioactive components of the ethanol-soluble fraction were analysed by paper chromatography and/or electrophoresis (Bernard *et al.*, 1993). The radioactive spots were visualized using a Packard Instant Imager. The radioactivity of the ethanol-insoluble fraction and the CO₂ trapped on filter paper soaked with 6 M KOH was also determined.

GB transmethylase assays. *P. aeruginosa* PAO1 strain was grown either in M63 with 10 mM GB as the carbon source, or in M63 with 10 mM glucose or 20 mM succinate as the carbon source, and with or without 1 mM GB. Cultures (100 ml) were incubated at 37 °C, with shaking (175 r.p.m.), and harvested by centrifugation at mid-exponential phase (OD₅₇₀ 0.7). The pellets were washed twice in carbon-free M63, once with sodium phosphate buffer (10 mM, pH 7.5), and finally resuspended in the same buffer to 100 OD₅₇₀ units ml⁻¹. Bacterial cells were disrupted with glass beads, and cell debris were removed by centrifugation; the clarified cell lysates were then used to carry out GB transmethylase assays using a procedure adapted from that performed by Serra *et al.* (2002). Briefly, the standard assay contained 10 mM Na⁺-phosphate buffer (pH 7.5), 50 µM L-homocysteine, 20 µM [methyl-¹⁴C]GB (2.83 GBq mmol⁻¹) and 0.5 mg protein in a final volume of 100 µl. The remaining substrate and the resulting products of this enzymic assay were extracted in 80% ethanol and analysed by TLC using aqueous phenol (80% w/v) as the mobile phase. The radioactive spots were visualized and quantified using a Packard Instant Imager.

Chemicals. The radiolabelled compound [methyl-¹⁴C]choline was purchased from NEN-Dupont. Glucose and succinate used in this study as carbon sources were purchased from Sigma-Aldrich. The osmoprotectants, GB, choline, L-carnitine, D, L-pipecolate, L-proline, sucrose and trehalose, were also purchased from Sigma-Aldrich. Ectoine was purchased from Bitop. Dimethylsulfonylacetate and dimethylsulfonylpropionate were synthesized in the laboratory as described previously (Pichereau *et al.*, 1998).

Separation of proteins by two-dimensional electrophoresis (2DE) and MALDI-TOF MS analysis. After harvesting by centrifugation, bacterial cells were washed in sodium phosphate buffer (10 mM, pH 7.5), then resuspended in the same buffer and disrupted by three passages through a French press. Cells debris was removed by centrifugation at 4 °C and 12 000 g for 30 min. The protein concentration of the supernatant fraction was determined according to the Lowry method. The lysate was precipitated with 10 % trichloroacetic acid, 80 % acetone, and then solubilized in the rehydration buffer (7 M urea, 2 M thiourea, 4 % CHAPS, 0.24 % Triton X-100, 20 mM DTT, 0.48 % Biolytes pH 3–10 and traces of bromophenol blue). For analytical and preparative two-dimensional (2D) gels, 200–300 µg crude protein extract was analysed by 2DE as previously described (Jebbar *et al.*, 2005). Proteins were identified by tryptic digestion of the polypeptides isolated from the Coomassie-stained control gels followed by peptide mass fingerprinting with a MALDI-TOF mass spectrometer (Voyager TM Elite; Perspective Biosystems) as described previously (Jebbar *et al.*, 2005). Peptide mass fingerprints were identified by comparison with theoretical values for proteins in the National Center for Biotechnology Information database or the *P. aeruginosa* protein database. The *P. aeruginosa* PAO1 protein database was created after downloading the whole-genome protein sequence of *P. aeruginosa* in FASTA format, available at the *Pseudomonas* genome project BLAST server (<http://www.pseudomonas.com/>). The proteins were identified by searching, in the above-cited databases, with the MS-FIT software of ProteinProspector (<http://prospector.ucsf.edu/>) and Mascot from Matrix Science (http://www.matrixscience.com/search_form_select.html). The search parameters allowed for oxidation of methionine, carbamidomethylation of cysteine and a maximum of one missed cleavage site within the peptides.

RESULTS

Influence of the carbon source on the osmoprotective effect of various compatible solutes in *P. aeruginosa*

To examine the influence of the carbon source on the osmoprotective effect of GB in *P. aeruginosa* PAO1 under conditions of high osmolality, cells were grown in M63 medium with or without 0.3, 0.5 or 0.75 M NaCl. Succinate

or glucose was used as the carbon source. GB was provided at a final concentration of 1 mM. Data presented in Table 1 show that succinate is the best carbon source at low salinity, but when medium osmolality rises glucose progressively becomes a better carbon source than succinate. At 0.75 M NaCl, PAO1 failed to grow on succinate, but it still grew on glucose. Addition of GB to M63 without NaCl did not modify the growth parameters, but it did improve growth when medium salinity increased from 0.3 to 0.75 M NaCl. The osmoprotective effect of GB on the growth rate was more obvious when succinate was used as the carbon source at 0.75 M NaCl in comparison to glucose. Various osmoprotectants were assayed to assess their ability to protect PAO1 cells from the detrimental effect of high salinity, when grown on succinate or glucose in M63 with 0.5 M NaCl. Only dimethylsulfoiniopropionate, dimethylsulfoacetate, ectoine and proline stimulated the growth as equally well as GB and its precursors choline and carnitine did (Lucchesi *et al.*, 1995), but other bacterial osmoprotectants such as pipercolate, sucrose and trehalose failed to protect PAO1 cells against osmotic stress (Table 2). At similar osmolalities the replacement of NaCl by the non-electrolyte sucrose did not induce significant changes in the growth pattern, but in the presence of GB, the GB osmoprotective effect was observable only at 0.96 M sucrose, a concentration that develops an osmolality equivalent to that of 0.75 M NaCl (data not shown).

Effect of the carbon source and GB on the nature of major accumulated osmolytes under hyperosmotic conditions

To determine the major osmolytes accumulated by *P. aeruginosa* at high osmolality, bacterial cells were grown in M63 medium containing 0.5 M NaCl with 20 mM succinate or 10 mM glucose, in the presence or the absence of 1 mM GB. Samples were harvested at mid-exponential and stationary phases. ¹³C NMR spectroscopy was then used

Table 1. Influence of carbon source and GB on growth of *P. aeruginosa* in media of various osmolalities

Cells were grown aerobically in M63 medium with 10 mM glucose or 20 mM succinate as a C source.

NaCl (M)	Result with the indicated osmoprotectant and carbon source*:							
	Glucose				Succinate			
	No osmoprotectant		1 mM GB		No osmoprotectant		1 mM GB	
	Growth rate (h ⁻¹)	Maximal OD ₅₇₀	Growth rate (h ⁻¹)	Maximal OD ₅₇₀	Growth rate (h ⁻¹)	Maximal OD ₅₇₀	Growth rate (h ⁻¹)	Maximal OD ₅₇₀
0	0.93	1.5	0.93	1.5	1.10	1.7	1.10	1.7
0.30	0.68	1.5	0.72	1.5	0.73	1.7	0.90	1.7
0.50	0.46	1.4	0.67	1.5	0.29	1.5	0.75	1.7
0.75	0.15	1.3	0.45	1.6	ND	ND	0.55	1.6

ND, Not detected.

*The values are the means of at least three independent experiments and the SD did not exceed 5 %.

Table 2. Effects of osmoprotectants on the growth of *P. aeruginosa* PAO1 at high salinity

Cells were grown aerobically in M63 with 10 mM glucose or 20 mM succinate as a C source.

NaCl (M) + osmoprotectant added to M63 culture medium*	Growth rate (h ⁻¹)†	
	Succinate	Glucose
0 + no osmoprotectant	1.10	0.93
0.5 + no osmoprotectant	0.29	0.46
0.5 + GB	0.75	0.67
0.5 + choline	0.69	0.66
0.5 + dimethylsulfoniopropionate	0.69	0.55
0.5 + dimethylsulfonioacetate	0.66	0.66
0.5 + carnitine	0.61	0.69
0.5 + ectoine	0.66	0.65
0.5 + proline	0.36	0.69
0.5 + pipecolate	0.28	0.44
0.5 + trehalose	0.29	0.43
0.5 + sucrose	0.28	0.45

*Each putative osmoprotectant was added to the culture medium at a final concentration of 1 mM.

†The values are means of at least two independent experiments and the SD did not exceed 5 %.

to identify all the solutes amassed to significant levels in the cells. The spectral data obtained with stressed cultures grown with or without GB are shown in Fig. 1. As described by D'Souza-Ault *et al.* (1993), the ¹³C NMR spectra showed that in the absence of exogenously provided osmoprotectant, cells synthesized and accumulated glutamate and the dipeptide NAGGN as the major organic osmolytes, and trehalose as a minor one (Fig. 1a). Similar results were obtained both with succinate-grown and glucose-grown cells, regardless of their growth stage. The spectral analysis of stressed cells in the presence of GB showed that when glucose was used as a carbon source, stressed cells from the late exponential growth phase accumulated GB and trehalose as minor organic osmolytes, and L-glutamate and NAGGN as the major ones (Fig. 1b). At stationary growth phase, signals from GB were undetectable, whereas those from trehalose, glutamate and NAGGN revealed high intracellular levels of these solutes (data not shown). When succinate was used as the carbon source, only minor amounts of glutamate and NAGGN, and no trehalose were detected; interestingly, exogenously supplied GB was by far the dominant cytoplasmic osmolyte at both exponential (Fig. 1c) and stationary growth phases (data not shown).

Uptake and fate of GB

To determine whether the strong difference of GB levels in succinate- and glucose-grown cells depended on GB uptake activity, we measured the influx of GB (10 µM) when cells were grown in M63 with increasing medium salinity, in the presence of succinate or glucose as the carbon source. The GB uptake activity of *P. aeruginosa* cells was 0.36 and 0.9 nmol min⁻¹ (OD unit)⁻¹ in M63 without NaCl,

supplemented with glucose and succinate, respectively. This activity increased to reach 2.8 nmol min⁻¹ (OD unit)⁻¹, when cells were grown in M63 with 0.3 M NaCl, and then decreased slightly to 2 nmol min⁻¹ (OD unit)⁻¹, when cells were grown in M63 with 0.5 M NaCl, whatever the carbon source used. The SD in the measurement of the transport rates did not exceed 5 %. Thus, these results suggest that the carbon sources used in this study did not affect the global GB uptake activity in *P. aeruginosa*, at least under stressing conditions.

Since ¹³C NMR spectroscopy can detect accumulated compounds only qualitatively, we determined by radiotracing experiments, through the whole growth cycle, the fate of 1 mM GB in *P. aeruginosa* cells cultivated at low (0 M NaCl) and high (0.5 M NaCl) salinities with glucose or succinate as the carbon source. Growth experiments showed that, in the presence of GB, cells grew better with succinate than with glucose at low salinity, as well as at high salinity (data not shown). As shown by the absence of both evolved radioactive CO₂ and insoluble materials, no GB degradation activity occurred when succinate was used as the carbon source at both low and high salinities during the exponential growth phase (Fig. 2b, d). Only a slight GB catabolic activity was detected at low salinity, when cells had reached the late stationary phase (Fig. 2b, d). Very low levels of GB were detected in the cells at low salinity, but at high salinity cells rapidly accumulated GB up to 560 nmol (OD unit)⁻¹ in early exponential growth phase. This level decreased to 470 nmol (OD unit)⁻¹ in late exponential phase. Less than 40 % of the total supplied radioactivity was taken up by the cells. The amount of GB decreased progressively to reach 280 nmol (OD unit)⁻¹, 2 h after the entry of the cells into the stationary growth phase. In parallel, the radioactivity (of

Fig. 1. Natural abundance ^{13}C -NMR spectrum of an ethanolic extract of *P. aeruginosa* PAO1 cells grown in M63 medium containing 0.5 M NaCl and 10 mM glucose or 20 mM succinate (a), 10 mM glucose with 1 mM GB (b) or 20 mM succinate with 1 mM GB (c). Resonances due to glutamate (gl), NAGGN (d), trehalose (t) and GB (gb) are indicated.

GB) in the supernatant increased at the entry of the cells into the stationary growth phase (Fig. 2a). Thus, a significant decrease in intracellular accumulated GB was not due to a start in a catabolic activity of this solute but rather to a GB efflux activity.

When glucose was used as the carbon source, GB degradation activity was detected at both low and high salinities. Samples were removed periodically, and the distribution of the radioactivity in the growth medium, the cellular ethanol-soluble fraction, the ethanol-insoluble fractions and $^{14}\text{CO}_2$ was determined as described in Methods. At low salinity, the majority of the radiocarbon was recovered in the $^{14}\text{CO}_2$ and in the ethanol-insoluble materials (Fig. 2b, d). At elevated salinity, the radioactivity in the medium decreased linearly and concomitantly with increasing cell density (Fig. 2a). The radioactivity incorporated by the cells was

Fig. 2. Effect of succinate or glucose on the fate of [methyl- ^{14}C]GB in salt-stressed or unstressed cells. *P. aeruginosa* PAO1 grown in M63 containing 1 mM [methyl- ^{14}C]GB, in the presence of 10 mM glucose (■, □) or 20 mM succinate (●, ○), without (filled symbols) or with 0.5 M NaCl (open symbols). Culture aliquots were withdrawn periodically to determine the remaining radioactivity in the supernatant (a), the radioactivity in the CO_2 (b), the radioactivity in the cytosol GB (c) and the radioactivity in the insoluble cellular macromolecules (d). Results were expressed as nmoles (OD unit) $^{-1}$ or nmoles ml^{-1} GB for cytosol GB (c) and supernatant (a), respectively. For the radioactivity recovered in CO_2 (b) and macromolecules (d), nmoles (OD unit) $^{-1}$ GB corresponds to the amount of catabolized GB allowing the production of a number of d.p.m. of radiolabelled carbons that were incorporated into macromolecules or released as $^{14}\text{CO}_2$. Results are the means of triplicate experiments; SE did not exceed 10%.

found in CO_2 , and in ethanol-insoluble and ethanol-soluble fractions. After two generations, more than 50 % of the total radioactivity was taken up by the cells and the radioactivity was mostly found in ethanol-soluble fraction and in $^{14}\text{CO}_2$ (Fig. 2b, c). Chromatographic and electrophoresis analyses of the ethanol-soluble fraction revealed that GB was the sole radiolabelled osmolyte, the maximal level reaching 520 nmol (OD unit) $^{-1}$ at early exponential growth phase; this level decreased to about 140 nmol (OD unit) $^{-1}$ in late exponential growth phase. Much more $^{14}\text{CO}_2$ was produced from GB at high salinity than at low salinity (Fig. 2b); the remaining radiocarbon was incorporated into insoluble

materials, and this fraction was much more labelled at low salinity than at high salinity (Fig. 2d).

The GB precursor choline was also assayed and the radio-tracing experiments carried out showed that choline was actively taken up and converted into GB at high salinity; the GB produced was subjected to CRC only when succinate but not glucose was used as the carbon source (data not shown). Unexpectedly, choline uptake was almost inactive at low salinity under our growth conditions, independent of the carbon source used (data not shown).

To determine if GB degradation activity was under the control of global regulators like Crc or Vfr, the fate of GB was also determined in *crc* and *vfr* mutant derivatives of wild-type *P. aeruginosa* PAO1. The results showed that GB degradation activity in these mutants was similar to that observed in PAO1, and this activity was repressed by succinate (data not shown).

Growth patterns of *P. aeruginosa* on GB plus succinate or glucose

In order to determine whether GB and glucose (or succinate) are used simultaneously or successively as carbon and energy sources, PAO1 cells were pre-cultured in minimal medium M63 with 20 mM succinate, and then used to inoculate M63 medium with 20 mM succinate, 5 mM succinate or a mixture of 5 mM succinate and 10 mM GB. A preculture in M63 with 10 mM glucose was used to inoculate M63 medium with 10 mM glucose, 2.5 mM glucose, or a mixture of 2.5 mM glucose and 10 mM GB. As a control, a pre-culture in M63 with 10 mM GB, was used to

inoculate M63 medium with the same carbon source. As shown in Fig. 3(a, b), the growth rate was slightly higher with succinate than with glucose. The maximal OD₅₇₀ reached was about 0.45 when succinate and glucose were used, at concentrations of 5 and 2.5 mM, respectively, while it reached 1.5 and 1.7 with 10 mM glucose and 20 mM succinate, respectively. The growth rate and growth yield in M63 with 10 mM GB as the sole carbon source were lower than those observed with 20 mM succinate and 10 mM glucose (Fig. 3a). These data showed that, in *P. aeruginosa*, the compatible solute GB was a less efficient carbon and energy source than succinate and glucose were. As expected, cells grown on a mixture of 2.5 mM succinate and 10 mM GB showed identical growth parameters as cells grown on 2.5 mM succinate without GB (Fig. 3b); but the cells grown on a mixture of succinate and GB restarted to grow and used GB after a lag of about 10 h, and after a short exponential growth phase the cells reached a maximal OD₅₇₀ of about 1 (data not shown). These data suggest that there was a long diauxic lag phase between the consumption of succinate and the initiation of GB utilization as a carbon source. However, growth on a mixture of glucose and GB resulted in a biphasic pattern characterized by two distinct exponential phases separated by a short lag phase of about 2 h (Fig. 3b). Thus, these results suggest that succinate repressed GB assimilation, but glucose had no effect even if we observed a diauxic-like lag.

Proteome pattern

To clarify the catabolite control of GB degradation, we prepared crude extracts of soluble proteins from *P. aeruginosa* PAO1 grown in M63 with glucose or succinate, with or without 1 mM GB. As a control, we also prepared crude extracts of proteins from cells grown in M63 with 10 mM GB as the sole carbon and energy source. The data obtained are presented in Fig. 4. By comparing the protein profile of GB-grown cells to the protein profiles of succinate- and glucose-grown cells, we observed that more than 20 proteins showed induced expression in the presence of GB (Fig. 4d). The 2DE gels obtained with succinate (20 mM) plus GB (1 mM) (Fig. 4a) and with succinate (20 mM) alone (not shown) were identical, and all proteins induced by GB in Fig. 4(d) were repressed in the presence of succinate. However, we observed a significant difference between 2DE gels from cells grown on glucose (10 mM) (Fig. 4b) and cells grown on glucose (10 mM) plus GB (1 mM) (Fig. 4c). A total of 11 of the GB-induced proteins, out of 21, were detected in Fig. 4(c). All of these protein spots were excised from the gels and examined by MALDI-TOF MS to generate mass fingerprints. The putative identities based on peptide mass fingerprint homology are given in Table 3. Among the identified polypeptides were SoxD (sarcosine oxidase δ subunit), GlyA1 and GlyA2 (serine-hydroxymethyltransferase), FdhA (glutathione-independent formaldehyde dehydrogenase), PurU2 (formyltetrahydrofolate deformylase), SdaB (L-serine dehydratase) and AdhC (glutathione-dependent formaldehyde dehydrogenase). The

Fig. 3. Growth of *P. aeruginosa* on glucose, succinate and GB. (a) Cells were grown in minimal medium M63 supplemented with (▲) 10 mM glucose, (□) 20 mM succinate or (△) 10 mM GB. (b) Cells were grown in M63 medium supplemented with 2.5 mM glucose (◇), 5 mM succinate (○), a mixture of 2.5 mM glucose and 10 mM GB (◆), or a mixture of 5 mM succinate and 10 mM GB (▲). Results are the means of at least three independent experiments; SE did not exceed 5%.

Fig. 4. Coomassie blue-stained 2DE gels of exponentially growing *P. aeruginosa* in M63 containing 20 mM succinate with 1 mM GB (a), 10 mM glucose (b), 10 mM glucose with 1 mM GB (c) or 10 mM GB (d). Equivalent amounts (300 μ g) of crude proteins were separated by 2DE. The proteins spots induced by GB, indicated by arrows, were analysed by MALDI-TOF MS and are listed in Table 2. The 2D gel experiments and MALDI-TOF analysis were carried out in triplicate and duplicate, respectively.

genes *glyA1*, *soxD*, *purU2* and *fdhA* encoding GB-induced proteins are organized in a gene cluster, which also contains *soxB*, *soxA* and *soxG* (<http://www.pseudomonas.com/>). All these enzymes belong to a probable sarcosine catabolism pathway similar to that described in *Arthrobacter* spp. (Meskys *et al.*, 2001).

In addition, proteins induced by GB also include those known as virulence factors, such as OprF (Sriramulu *et al.*, 2005), an outer-membrane protein and a phosphorylcholine phosphatase enzyme (Fig. 4d, Table 3), which has been described as an enzyme produced in the presence of choline, GB, carnitine or dimethylglycine in *P. aeruginosa* (Massimelli *et al.*,

Table 3. *P. aeruginosa* GB-induced proteins characterized by MALDI-TOF MS

Spot no.	Calculated M_r /pI*	Theoretical M_r /pI†	Mouse score‡	No. of peptides matched§	Sequence coverage (%)‡	Protein name	Genomic accession no.
1	46·48/4·90	39·76/5·10	1·04E+10	25/114	54	Hypothetical protein	PA3515
2	65·91/5·37	53·14/5·41	4·36E+11	25/150	59	Probable aldehyde dehydrogenase	PA5312
3	58·67/5·48	40·53/5·51	9·72E+09	20/150	58	Probable ferredoxin	PA5411
4	74·04/5·58	49·21/5·62	1·52E+08	22/150	39	L-serine dehydratase	PA5379
5	51·02/5·41	45·40/5·48	3·96E+06	13/150	30	Probable aminotransferase	PA5523
6	47·58/5·39	39·80/6·20	1·48E+10	25/77	56	Probable binding protein component of ABC transporter	PA4913
7	41·37/5·48	39·11/5·68	7·21E+09	24/150	55	Phosphorylcholine phosphatase	PA5292
8	54·71/6·00	44·70/6·12	8·45E+08	28/150	59	Serine hydroxymethyltransferase	PA5415
	54·71/6·00	44·97/6·07	1·18E+06	18/150	35	Serine hydroxymethyltransferase	PA2444
9	48·70/6·11	42·00/6·27	3·34E+17	36/150	71	Glutathione-independent formaldehyde dehydrogenase	PA5421
10	40·42/5·90	37·52/5·97	5·28E+11	27/150	81	Hypothetical protein	PA3518
11	27·21/5·86	34·42/6·76	2·06E+10	22/139	62	Probable GB/proline transport system substrate-binding protein	PA5378
12	32·78/5·41	36·08/5·47	5·65E+11	29/136	67	Hypothetical protein	PA5396
13	21·06/5·41	23·78/5·48	8·32E+10	22/150	83	Probable glutathione S-transferase	PA4401
14	26·59/6·35	31·08/8·40	7·34E+08	21/150	61	Probable GB-binding protein precursor	PA3236
15	6·73/4·85	13·02/5·06	6·86E+03	8/150	58	Hypothetical protein	PA2762
16	40·90/4·72	37·64/4·98	1·31E+04	12/150	35	Outer-membrane protein OprF precursor	PA1777
17	39·49/4·72	37·12/4·78	4·45E+08	17/150	44	Phosphoribosylaminoimidazole synthetase	PA0945
18	09·55/5·32	12·14/5·90	5·46E+02	6/150	57	Sarcosine oxidase δ subunit	PA5417
19	51·02/5·53	39·21/5·47	2·98E+05	11/120	41	Glutathione-dependent formaldehyde dehydrogenase	PA3629
20	33·55/5·44	33·48/5·40	7·57E+04	10/120	34	Thioredoxin reductase 2	PA0849
	33·55/5·44	35·37/5·46	1·74E+04	8/120	27	Homoserine kinase	PA5495
21	29·18/6·23	32·29/6·15	6·16E+06	15/120	60	Formyltetrahydrofolate deformylase	PA5420

*Observed molecular mass and isoelectric point obtained from 2DE calibrated with known standards (Bio-Rad). M_r is in thousands.

†Theoretical M_r and pI calculated for predicted amino acid sequence of the protein. M_r is in thousands.

‡Mouse score and sequence coverage are indicated in order to facilitate judgement of the reliability of the data.

§Number of peptides that matched with molecular mass of theoretical trypsin-digested peptides of *P. aeruginosa* proteins.

||The protein name and corresponding gene number in the genome of *P. aeruginosa*, based on the *P. aeruginosa* genomic database (<http://www.pseudomonas.com/>).

2005). Among the GB-induced proteins, there was a putative GB/proline transport substrate-binding protein (PA5378), a component of an ABC-type transporter homologue to ProU from *Escherichia coli* (Lucht & Bremer, 1994) and OpuC from *Bacillus subtilis* (Kappes *et al.*, 1996). This system was renamed Opu, for osmoprotectant uptake, and the genes *pa5378*, *pa5377* and *pa5376*, composing this system, were renamed *opuA*, *opuB* and *opuC*, respectively. Furthermore, many other proteins with undefined biochemical functions were synthesized by the cells in the presence of GB (Table 3). When glucose was used as the carbon source, the addition of GB allowed the induction of some GB-induced proteins that did not comprise SdaB, SoxD, PurU2 and AdhC proteins (Fig. 4c); this suggests that these enzymes are not implicated in GB degradation in the presence of glucose, and that the other proteins present on 2D gels could be sufficient for the GB catabolism activity

described above in Fig. 2. In contrast, all GB-induced proteins were undetectable in the presence of both succinate and GB. These data confirm the radiotracing data described above and demonstrate that succinate represses the synthesis of enzymes involved in GB degradation.

GB transmethylation activity

To assay *in vitro* the GB transmethylation activity, cells were grown in M63 with 10 mM glucose, 10 mM glucose plus 1 mM GB, 20 mM succinate, 20 mM succinate plus 1 mM GB, or 10 mM GB. Cultures were harvested at mid-exponential phase, and crude extracts were prepared and checked for their ability to catabolize [*methyl*- 14 C]GB in the presence of L-homocysteine. Curiously, no GB catabolic activity was detected in crude protein extracts from cells grown in M63 with glucose as a carbon source and 1 mM

Fig. 5. Enzymic degradation of [methyl- ^{14}C]GB by crude extracts from *P. aeruginosa*. Cells were grown to mid-exponential phase in M63 medium with various carbon sources as described in Methods, harvested by centrifugation, and washed twice with carbon-free M63 medium. Bacterial cells were resuspended in sodium-phosphate buffer (10 mM pH 7.5), and disrupted with glass beads; the resulting crude extracts were used for enzymic assays. After 15 min, the reaction mixtures were extracted with 80% ethanol, and soluble metabolites were analysed by TLC using phenol as a mobile phase. Lanes 1 to 6 correspond to the enzymic activity of crude extracts from cultures grown in M63 with the indicated carbon source(s): 1, radiolabelled GB as a standard; 2, 10 mM betaine; 3, 10 mM glucose; 4, 10 mM glucose + 1 mM betaine; 5, 20 mM succinate; 6, 20 mM succinate + 1 mM betaine. GB, glycine betaine; DMG, dimethylglycine; Met, methionine; Sar, sarcosine; Ser, serine. Experiments were carried out in triplicate.

GB (Fig. 5). Such an activity was observed only in the crude extract from culture grown in M63 with 10 mM GB as a carbon source (Fig. 5). Surprisingly, we were able to detect not only radioactive methionine and dimethylglycine, which are produced by a GB transmethylase (Gbt) activity reported in *P. aeruginosa* (Serra *et al.*, 2002), but also catabolic products such as sarcosine and serine. These data showed that enzymes such as sarcosine oxidase and serine hydroxymethyltransferase, in addition to Gbt, are still active *in vitro*.

DISCUSSION

Osmoprotectants have been considered for a long time as metabolically inert compounds under high osmolality conditions. The most potent osmoprotectant GB and some structurally related compounds, such as choline and carnitine, are known to serve as both carbon and nitrogen sources, but only at low osmolality (Lucchesi *et al.*, 1995; Serra *et al.*, 2002; Smith *et al.*, 1988). However, the alfalfa

symbiont *S. meliloti* can use GB as both an osmoprotectant and a source of carbon and energy (Talibart *et al.*, 1997), leading the dissimilative pathway for GB to be operative at both high and low osmolalities in this bacterium (Talibart *et al.*, 1997). In this study, we showed that besides GB itself (D'Souza-Ault *et al.*, 1993), only its immediate precursors (choline and carnitine) (Lucchesi *et al.*, 1995), two of its sulfonium structural analogues (dimethylsulfonylacetate and dimethylsulfonylpropionate), ectoine and proline were efficient osmoprotectants for *P. aeruginosa* PAO1 (Table 2). Even pipercolate, which is known to protect efficiently various bacteria against hyperosmotic constraints (Choquet *et al.*, 2005; Gouesbet *et al.*, 1994; Gouffi *et al.*, 2000), has no beneficial effect on growth of *P. aeruginosa* at high salinity (Table 2). As described previously in *S. meliloti* (Talibart *et al.*, 1997), we showed that GB was only transiently accumulated in *P. aeruginosa*. Moreover, the ability of PAO1 to degrade GB was repressed in the presence of succinate at both low and high osmolalities. *P. aeruginosa* exhibits diauxic growth in media containing both GB and succinate; although in this case succinate was utilized first, while GB catabolism occurred several hours after complete succinate consumption. The presence of a long and unusual lag phase could be explained by a need to induce enzymes involved in GB degradation, and to adapt the cellular machinery to produce energy from catabolic products resulting from GB degradation. When used as a carbon and energy source, GB could support growth but not as efficiently as succinate and glucose did (Fig. 3a). In addition, when M63 containing 10 mM GB (as the carbon source) was inoculated with pre-cultures grown on succinate or glucose, PAO1 cells started to grow after a lag phase of at least 7 h (data not shown). This long lag phase was apparently required to initiate the catabolic and energy production machinery necessary to assimilate GB. *P. aeruginosa* PAO1 is known to exhibit diauxic growth when it is cultured on succinate and other tricarboxylic acid cycle intermediates in the presence of carbohydrate compounds such as glucose, mannitol, gluconate, glycerol, glycerate or fructose (Collier *et al.*, 1996). In addition to enzymes of the carbohydrate catabolic pathways of *P. aeruginosa*, succinate mediates repression of several other catabolic pathways involved in the utilization of histidine, methylamine and arginine (Collier *et al.*, 1996). The Crc protein, which is involved in the catabolic repression by succinate of the expression of several genes implicated in the metabolism of some carbohydrates and nitrogenous compounds, is not often necessary for mediating CRC of all genes and operons in *P. aeruginosa* (Collier *et al.*, 1996). Succinate, via a CRC mechanism that does not involve Crc protein, directly regulates osmoprotectant-dependent expression of *plcH*, encoding the haemolytic PLC (Sage & Vasil, 1997; Sage *et al.*, 1997). Since *P. aeruginosa* utilizes GB, and its precursors choline and carnitine, as sole carbon and energy sources, it seems possible that succinate represses the degradation of GB (this study), which results in a repression of osmoprotectant-dependent *plcH* expression in *P. aeruginosa* PAO1 (Sage & Vasil, 1997).

Growth on a combination of GB and glucose showed a diauxic-like profile; from this observation, one could speculate that glucose may exert CRC control on GB utilization. This could not be the case, however, because the delay necessary for the initiation of growth on GB is slightly longer than that allowing the full utilization of glucose. Furthermore, the radiotracing experiments (Fig. 2) showed that cells degraded GB at low and high salinities in the presence of glucose; they also showed that for the crude extracts prepared from cells grown on a combination of glucose and GB as carbon and energy sources (presenting a diauxic-like growth profile), a GB degradation activity occurred from a lag and second exponential growth phase (Fig. 3b) but not from the first exponential growth phase (data not shown). In contrast, the absence of GB-demethylating activity in crude extracts from cells grown in M63 with glucose as the carbon source and 1 mM GB (Fig. 5) could be linked to the absence of 10, out of 21, proteins induced by GB (Fig. 4c, d). The other explanations are that (i) at 1 mM, in comparison to 10 mM, GB is probably not sufficient to induce enough GB catabolic enzymes necessary to detect GB-demethylating activity *in vitro*, or (ii) all GB catabolic enzymes (necessary when GB is used as carbon source) are probably not required to degrade 1 mM GB in the presence of a carbon and energy source such as glucose. This implies that the glucose catabolic pathway(s) helps to assimilate GB under these conditions.

Our proteomic study showed that GB, when used as a carbon and energy source, induced proteins involved in its own degradation, and that the induction of these proteins was subjected to CRC generated by succinate. The radiotracing studies allowed detection of radiolabelled dimethylglycine, methionine, sarcosine and serine (Fig. 5), which are the catabolic products of [*methyl*-¹⁴C]GB. Thus, GB was demethylated in three steps to produce glycine, and dimethylglycine and sarcosine as intermediates; sarcosine oxidase and other undetected enzymes, such as Gbt and dimethylglycine demethylase or dehydrogenase, are involved in this catabolic pathway. Glycine could be further transformed, probably through the action of hydroxymethyltransferase (GlyA1 and GlyA2) into serine, which in turn could give pyruvate through serine dehydratase (SdaB) activity. The *P. aeruginosa* PAO1 genome also contains *sdaA* (PA2443), a gene encoding another serine dehydratase that, curiously, was not detected on our 2DE; *sdaA* belongs to the same gene cluster as *glyA2* (PA2444) whose product was detected as being a protein accumulated when GB was used as a bacterial growth substrate. Sarcosine oxidase characterized both in *Arthrobacter* spp. (Meskys *et al.*, 2001) and *Corynebacterium* sp. P-1 (Chlumsky *et al.*, 1995) is a heterotetrameric enzyme composed of β , δ , α and γ subunits encoded, respectively, by genes *soxB*, *DAG* that are organized in an operon with *glyA* and *purU2* genes, themselves encoding a serine hydroxymethyltransferase and a formyltetrahydrofolate deformylase, respectively. The genetic organization of this cluster composed of genes encoding enzymes for the catabolism of sarcosine to glycine in *Arthrobacter* spp. and *Corynebacterium* sp. P-1 is found to

be similar to that of *P. aeruginosa* (Stover *et al.*, 2000), but different in that the glutathione-independent formaldehyde dehydrogenase (FdhA) gene was observed downstream of *purU2* in *P. aeruginosa* (Stover *et al.*, 2000).

It has been demonstrated that sarcosine oxidase from *Corynebacterium* sp. P-1 and *Arthrobacter* spp. catalyses the oxidative demethylation of sarcosine to yield glycine, H₂O₂ and 5,10-methylenetetrahydrofolate, in the presence of tetrahydrofolate and O₂ (Chlumsky *et al.*, 1995; Meskys *et al.*, 2001). In the absence of tetrahydrofolate, the same rate of sarcosine oxidation is observed and the oxidized methyl group is released as formaldehyde (Chlumsky *et al.*, 1995; Meskys *et al.*, 2001). Sarcosine oxidase enzymes from *P. aeruginosa*, *Corynebacterium* sp. P-1 (Chlumsky *et al.*, 1995) and *Arthrobacter* spp. (Meskys *et al.*, 2001) each contain a putative folate binding domain. Also, enzymes that utilize folates as substrates, such as PurU2, a 10-formyltetrahydrofolate deformylase, and GlyA1 and GlyA2, two hydroxymethyltransferase enzymes, were induced during growth of *P. aeruginosa* on GB. These observations further suggest that in *P. aeruginosa* tetrahydrofolate is involved in GB catabolism as a C1 acceptor. In this study, different enzymes that could use formaldehyde as substrate were induced in *P. aeruginosa* in the presence of GB (Table 2, Fig. 4d). It seems that GB degradation, via a sarcosine oxidase activity, could produce formaldehyde, which in turn could be used by FdhA (PA5421) and AdhC (PA3629) to produce formate. Tracing experiment using radiolabelled GB showed that high amounts of radioactive CO₂ were produced, suggesting that formate was transformed into CO₂ and H₂O by a probable formate dehydrogenase, which was not detected as an accumulated protein on our 2D gels. Serine dehydratase (SdaB), cited above, is an enzyme encoded by *sdaB* gene. This gene, while oriented divergently, is adjacent to *opuA* (pa5378), which encodes a putative binding protein belonging to a GB/proline transport system that was also induced in the presence of GB. The adjacent localization of the two genes suggests that their simultaneous induction by GB could be dependent on the same regulatory mechanism. The *opuA* gene belongs to an *opu* operon composed of three genes (*opuABC*) encoding components of an ABC-type transporter. Usually, the ABC-type transporters of osmoprotectants are induced by elevated osmolality but not by their substrates (Bremer & Krämer, 2000; Choquet *et al.*, 2005), except Hut and Ehu transporters from *S. meliloti*, which are induced by histidine and ectoines (ectoine and hydroxyectoine), respectively (Boncompagni *et al.*, 2000; Jebbar *et al.*, 2005).

All these data led us to propose a reconstructed GB catabolic pathway in *P. aeruginosa* (Fig. 6), which is similar to that proposed in *Arthrobacter* spp. (Meskys *et al.*, 2001) and *S. meliloti* (Smith *et al.*, 1988).

In conclusion, GB operates as both a carbon source and an osmoprotectant in glucose-grown PAO1 cells, but only as an osmoprotectant in succinate-grown cells. The different patterns of GB utilization in the presence of glucose and

Fig. 6. The proposed GB catabolic pathway in *P. aeruginosa* reconstructed on the basis of proteomic and metabolic data presented in this study. Opu, osmoprotectant uptake; Gbt, GB transmethylase; Sox, sarcosine oxidase; GlyA, serine hydroxymethyltransferase; SdaA and SdaB, serine dehydratase; PurU2, 10-formyltetrahydrofolate deformylase; FdhA, glutathione-independent formaldehyde dehydrogenase. AdhC, glutathione-dependent formaldehyde dehydrogenase. All proteins labelled in bold were identified in this study.

succinate are linked to the fact that succinate apparently represses the synthesis of enzymes involved in GB catabolism. Genetic and biochemical characterization, especially of those genes and proteins with unknown biochemical function, will help to elucidate the regulation of GB catabolism by succinate, and to establish GB utilization pathway(s) and the physiological conditions under which it (or they) function in *P. aeruginosa*.

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