Microbiology (2011), **157**, 1531–1540 DOI 10.1099/mic.0.047191-0

Repression of the glucose-inducible outer-membrane protein OprB during utilization of aromatic compounds and organic acids in *Pseudomonas putida* CSV86

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Received 30 November 2010
Revised 8 February 2011
Accepted 15 February 2011

Pseudomonas putida CSV86 shows preferential utilization of aromatic compounds over glucose. Protein analysis and [^{14}C]glucose-binding studies of the outer membrane fraction of cells grown on different carbon sources revealed a 40 kDa protein that was transcriptionally induced by glucose and repressed by aromatics and succinate. Based on 2D gel electrophoresis and liquid chromatography-tandem mass spectrometry analysis, the 40 kDa protein closely resembled the porin B of *P. putida* KT2440 and carbohydrate-selective porin OprB of various *Pseudomonas* strains. The purified native protein (i) was estimated to be a homotrimer of 125 kDa with a subunit molecular mass of 40 kDa, (ii) displayed heat modifiability of electrophoretic mobility, (iii) showed channel conductance of 166 pS in 1 M KCl, (iv) permeated various sugars (mono-, di- and tri-saccharides), organic acids, amino acids and aromatic compounds, and (v) harboured a glucose-specific and saturable binding site with a dissociation constant of 1.3 μM. These results identify the glucose-inducible outer-membrane protein of *P. putida* CSV86 as a carbohydrate-selective protein OprB. Besides modulation of intracellular glucose-metabolizing enzymes and specific glucose-binding periplasmic space protein, the repression of OprB by aromatics and organic acids, even in the presence of glucose, also contributes significantly to the strain's ability to utilize aromatics and organic acids over glucose.

INTRODUCTION

The most effective and economical way to remove aromatic pollutants is by means of microbial degradation (Alexander, 1981). The metabolism of individual aromatic compounds by micro-organisms has been extensively studied. However, studies on the response of micro-organisms to a mixture of aromatic compounds are scarce (Haigler *et al.*, 1992; Higgins & Mandelstam, 1972). Due to the potential application of these strains in bioremediation, there are increasing reports on isolates that can utilize mixtures of aromatic compounds, particularly in the presence of glucose. In nature, micro-organisms show a preference for carbon sources like glucose and organic acids. Unless these are depleted, microbes do not degrade toxic and mutagenic compounds like aromatics due to carbon catabolite repression (CCR) which is established in several micro-organisms (Collier *et al.*, 1996). A well-studied example is the repression of lactose utilization by glucose in *Escherichia coli* (Stülke & Hillen, 1999). In pseudomonads, glucose utilization was found to be repressed by organic acids, known as reverse CCR (Collier *et al.*, 1996). Although CCR is not well studied in pseudomonads, glucose and organic acids have been reported to repress the enzymes and transport system involved in the utilization of several aromatic compounds (Duetz *et al.*, 1994; Holtel *et al.*, 1994; McFall *et al.*, 1997; Müller *et al.*, 1996; Ng & Dawes, 1973; Schleissner *et al.*, 1994; Tiwari & Campbell, 1969).

In *Pseudomonas*, glucose uptake occurs via either oxidative (low affinity, K_m 1–2 mM) or phosphorylative (high affinity, K_m 8 μM) pathways (Hancock & Carey, 1980; Midgley & Dawes, 1973; Wylie *et al.*, 1993). The oxidative pathway involves two different routes of glucose metabolism. Glucose can be converted to gluconate and 2-ketogluconate by glucose dehydrogenase and 2-ketogluconate dehydrogenase, respectively, in the periplasmic space. Alternatively, gluconate or 2-ketogluconate can be

Abbreviation: LC-MS/MS, liquid chromatography-tandem mass spectrometry.

transported into the cytoplasm through two different transporters and converted to 6-phosphogluconate (6-PG). In the phosphorylative pathway, glucose is transported across the cytoplasmic membrane and metabolized to 6-PG by the action of glucokinase and glucose-6-phosphate dehydrogenase (Zwf) in the cytoplasm. 6-PG, the key intermediate, enters the TCA cycle via glyceraldehyde-3-phosphate and pyruvate through the Entner–Doudoroff pathway (Daddaoua *et al.*, 2009).

In *Pseudomonas putida* KT2440, glucose metabolism was found to be repressed by toluene. However, this catabolite repression was not as strict as reported in cases of glucose utilization over lactose in *E. coli*, and the organism utilizes glucose and toluene simultaneously, counterbalancing the total carbon uptake (del Castillo & Ramos, 2007). In strain KT2440, toluene represses the metabolism of glucose at the level of glucokinase, which is mediated through Crc, a regulator. On the other hand, glucose suppresses the toluene metabolism through 2-keto-3-deoxy-6-phosphogluconate (KDPG), which acts as a signal molecule to enable the repression. KDPG has also been shown to act as a signal molecule in the repression of phenylacetic acid metabolism (Kim *et al.*, 2009). Transcriptomic studies from glucose + toluene-grown cells of strain KT2440 demonstrate the down-regulation of genes involved in the glucose transport (OprB channel, and components of the ABC transport system). However, toluene did not affect the oxidative route of glucose metabolism (del Castillo & Ramos, 2007). The phosphorylative mode of glucose metabolism requires its transport inside the cell and involves: (i) an inducible outer-membrane protein (Hancock & Carey, 1980; Saravolac *et al.*, 1991; Wylie & Worobec, 1995), (ii) an inducible periplasmic glucose-binding protein (Basu *et al.*, 2007; Sly *et al.*, 1993; Stinson *et al.*, 1977) and (iii) a putative inner-membrane protein(s) (Adewoye & Worobec, 2000). So far, to our knowledge, all three components have not been reported from a single *Pseudomonas* strain.

The channel-forming proteins present in the outer membrane of Gram-negative bacteria, generally termed as porins, allow diffusion of nutrients, metabolites and toxic waste across the outer membrane (Hancock & Carey, 1980; Nikaido, 2003). Porins are of two types: (i) non-specific porins which allow diffusion of substrates on the basis of mass, charge and polarity, e.g. OmpF of *E. coli* (Cowan *et al.*, 1992) or OprF of *Pseudomonas aeruginosa* (Nestorovich *et al.*, 2006; Sugawara *et al.*, 2006), and (ii) specific channels which show high-affinity substrate transport and often possess a binding site for a given substrate within the channel e.g. maltodextrin-specific LamB of *E. coli* (Benz *et al.*, 1987), glucose-specific OprB of *P. aeruginosa* and *P. putida* (Hancock & Carey, 1980; Saravolac *et al.*, 1991; Trias *et al.*, 1988; Wylie *et al.*, 1993), imipenem- and basic amino-acid-specific OprD (Trias & Nikaido, 1990) and phosphate-specific OprP (Hancock *et al.*, 1982; Hancock & Benz, 1986) of *P. aeruginosa.*

P. putida strain CSV86 (hereafter referred to as CSV86) is a soil isolate that utilizes aromatic compounds or organic acids in preference to glucose (Basu *et al.*, 2006). In CSV86, glucose is taken up by active transport and is metabolized via an inducible intracellular phosphorylative pathway and not by an oxidative pathway (Basu & Phale, 2006). A 43 kDa periplasmic glucose-binding protein has earlier been reported to be induced during growth on glucose and suppressed during growth on aromatics and organic acids (Basu *et al.*, 2007). The present study investigates the outermost transport component and its possible involvement in such regulation. Here, we report the induction of a 40 kDa outer-membrane protein in CSV86 by glucose and repression by aromatics and organic acids, and its identification as an OprB channel based on proteomic, biochemical and functional studies. These results suggest the repression of OprB and other glucose transport components by aromatics and organic acids may facilitate their preferential utilization over glucose.

METHODS

Growth conditions and extraction of outer-membrane proteins. *P. putida* CSV86 (Mahajan *et al.*, 1994) was grown on minimal salt medium (MSM, 150 ml) in 500 ml baffled Erlenmeyer flasks at 30 °C on a rotary shaker (200 r.p.m.). Medium was supplemented aseptically with the appropriate aromatics (0.1 %), glucose (0.25 %) or organic acid (0.25 %) either singly or in combination as the source of carbon and energy, as described earlier by Basu *et al.* (2003). Growth was monitored at 540 nm using MSM as blank. The inoculum volume from preinoculum grown on aromatics (7 h), glucose (22 h) and organic acid (7 h) was 2 ml. Outer-membrane proteins were extracted as described by Nakajima *et al.* (1998) with minor modifications: cells grown on an appropriate carbon source(s) were harvested, washed with Buffer A (Tris/HCl 10 mM, pH 8.0 and EDTA 10 mM), suspended (1 g wet weight in 5 ml) in the same buffer and disrupted by French press (two cycles at 12 000 p.s.i.; Thermoelectron). Unbroken cells were removed by centrifugation at 10 000 ***g*** for 10 min, and supernatant was further centrifuged at 50 000 ***g*** for 60 min. The membrane pellet, thus obtained, was washed with NaCl (0.3 M in Buffer A), centrifuged and suspended in *N*-lauroylsarcosine (1 % in Buffer A, 2 ml g^{-1}). The suspension was stirred at 30 °C for 40 min and centrifuged at 50 000 ***g*** for 60 min. The resulting pellet containing the insoluble outer membrane fraction was suspended in a minimum amount of Buffer A and studied further. Protein content was estimated by using the method of Bradford (1976) using BSA as the standard.

Electrophoretic resolution and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of outer-membrane proteins. Outer-membrane proteins were resolved by SDS-PAGE (10 %) as described by Laemmli (1970). Outer-membrane protein fractions were also subjected to 2D gel electrophoresis (Basu *et al.*, 2007; Joshi *et al.*, 2004; O'Farrell, 1975) followed by LC-MS/MS analysis (ESI-TRAP, trypsin digest; peptide mass tolerance ± 2 Da; fragment mass tolerance, 0.8 Da) as described by Basu *et al.* (2007). The data were analysed by using the Mascot protein identification system (Matrix Science) with the database from The Institute of Genomic Research (TIGR; http://www.jcvi.org/). The criterion for reliable identification was a significant Mascot score, >47 ($P<0.05$).

[^{14}C]Glucose-binding assay. [^{14}C]Glucose-binding activity was measured after dialysing the outer-membrane fraction against

Buffer B (Buffer A containing 0.1 % Triton X-100) by using the method described by Sly *et al.* (1993). The assay mixture (1 ml) contained dialysed outer-membrane protein fraction (100 μg), [^{14}C]glucose [500 nM, 140 mCi mmol^{-1} (5.1 GBq mmol^{-1}), BRIT-India] and Buffer B. The mixture was incubated for 5 min at 30 °C and rapidly filtered through pre-moistened 0.45 μm PVDF membranes (Pall Life Sciences). Filters were washed with Buffer B (1 × 2 ml), air-dried and vigorously mixed in scintillation cocktail (0.4 % PPO and 0.025 % POPOP in toluene). Radioactivity was measured using a liquid scintillation counter (LKB). Glucose-binding activity was expressed as pmol bound [^{14}C]glucose (mg protein)$^{-1}$.

Purification of glucose-inducible outer-membrane protein. The outer-membrane proteins were extracted by suspending the *N*-lauroylsarcosine-insoluble membrane fraction in Buffer B (membrane pellet obtained from 1 g wet weight cells in 5 ml) for 5 h at 30 °C on a rotary shaker. The mixture was centrifuged at 50 000 ***g*** for 60 min. The supernatant containing outer-membrane protein was loaded onto DEAE-Sephacel ion exchange column (120 × 12 mm, bed vol. 13 ml) pre-equilibrated with Buffer B. The column was washed with NaCl (50 mM) in Buffer B (100 ml). The bound protein was eluted with a linear gradient of NaCl (50–400 mM) in Buffer B (150 ml, 2.5 ml fraction, flow rate 30 ml h^{-1}). Fractions were analysed by using SDS-PAGE and [^{14}C]glucose-binding assay. Fractions containing 40 kDa protein and [^{14}C]glucose-binding activity were pooled, concentrated and resolved by Sephacryl S-200-HR gel filtration column chromatography (860 × 16 mm, bed vol. 170 ml, void vol. 59 ml) equilibrated with Buffer B containing NaCl (150 mM). Fractions (1 ml, flow rate of 8 ml h^{-1}) containing the 40 kDa protein and glucose-binding activity were pooled, concentrated and stored at 4 °C in Buffer B.

Determining channel conductance. Planar lipid bilayers were assembled from monolayers by a modification of the technique described by Montal & Mueller (1972). Briefly, a 100 μm aperture in a thin Teflon membrane separating two Teflon chambers was primed with hexadecane in *n*-pentane (1 : 1, v/v). Monolayers of DPhPC (diphytanoyl-phosphatidylcholine, 10 mg ml^{-1}) were spread on KCl (1 M) in HEPES (5 mM, pH 7.4) solutions in both chambers. Bilayers were formed by slowly lowering and raising the solution level past the aperture. Bilayer formation was followed by monitoring conductance and capacitance. Voltage-clamp recording was performed using a BC-525C bilayer clamp amplifier (Warner Instruments). Analogue data from the amplifier were filtered at 1 kHz and digitized with Digidata 1322 (Axon Instruments/Molecular Devices) at 10 kHz. The pCLAMP 9.2 (Axon Instruments) software package was used to generate voltage-clamp commands, acquire membrane currents and analyse digitized data. Purified outer-membrane protein (10 ng ml^{-1}) was added to the *cis* chamber and channel conductance was monitored at voltages ranging from −120 mV to +120 mV. Slope conductances were estimated by plotting measured currents through single channels against applied voltage.

Liposome swelling assay. Purified glucose-inducible outer-membrane protein was reconstituted into proteoliposomes as described by Nikaido & Rosenberg (1983) with minor modifications. DOPC (dioleyl-phosphatidylcholine; 20 mg ml^{-1}) and EPG (eggphosphatidyl glycerol, 1 mg ml^{-1}) were dissolved in chloroform in a round bottomed flask. The solvent was evaporated by rotavaporator (Buchi) for 30 min at 40 °C. Phospholipids were hydrated in imidazole/NAD (1 mM, pH 6.0) and an aqueous suspension of either outer-membrane extract (200 μg) isolated from cells grown on different carbon sources or purified glucose-inducible outer-membrane protein (50 μg) in a total volume of 2 ml for 45 min at 40 °C. The suspension was sonicated (two cycles, each cycle 120 W for 1 min and 30 s interval) in a water bath sonicator until the suspension became a homogeneous translucent mixture with turbidity indicating the conversion of most liposomes into vesicles. The suspension was dried under vacuum at 50 °C and hydration was completed by adding stachyose (12 mM), sodium/NAD (4 mM) and imidazole/NAD (1 mM, pH 6.0). The solution was finally sonicated (2 min) and incubated at 30 °C for 2 h. Control liposomes were prepared in a similar fashion but without protein. The isosmotic concentration of each test solute prepared in imidazole/NAD (1 mM, pH 6.0) was determined by diluting control liposome preparation into different concentrations of test solutes and monitoring the changes in the light scattering at 500 nm (Perkin Elmer Lambda 35). The concentration of solute at which no time-dependent changes in A_{500} were observed was considered to be the isosmotic concentration (Sundara Baalaji *et al.*, 2006). The swelling of proteoliposomes was monitored by diluting them into isosmotic concentrations of various solutes in imidazole/NAD buffer and recording the rate of decrease of A_{500}. The initial rate of swelling (0–10 s) was considered to calculate the rate of permeation of solutes as decrease in A_{500} min^{-1}. Relative rates of permeation for other solutes were calculated taking the permeation rate for glucose as 100 %.

For determination of specificity, the assay mixture (1 ml) contained purified 40 kDa protein (20 μg), [^{14}C]glucose (500 nM) and 100-fold molar excess (50 μM) of unlabelled sugars, organic acids or aromatic compounds. Naphthalene was dissolved in DMSO, while all other compounds were dissolved in Buffer A. Control reactions contained [^{14}C]glucose, protein, Buffer A and an appropriate amount of DMSO. The reaction mixture was incubated for 5 min at 30 °C and rapidly filtered through pre-moistened 0.45 μm PVDF membranes. Radioactivity was measured as described above and expressed as per cent activity of [^{14}C]glucose binding, taking the binding for glucose as 100 %.

All experiments described were performed at least three times. The mean (±SD) values are presented where appropriate. The trends observed in SDS-PAGE and 2D-gel profiles were identical; the best profiles are shown.

RESULTS

Induction of the 40 kDa outer-membrane protein, [^{14}C]glucose binding and liposome swelling assay

When grown on glucose, CSV86 displayed a prominent protein band corresponding to 40 kDa which was absent when cells were grown on naphthalene (Fig. 1a), succinate (Fig. 1b) or benzyl alcohol (data not shown). The outer-membrane protein extracted from glucose-grown cells showed a higher [^{14}C]glucose-binding activity of 45–50 pmol (mg protein)$^{-1}$ compared with that of cells grown on naphthalene (0.9), succinate (1.2) or benzyl alcohol (0.2). When naphthalene-grown culture was inoculated into glucose medium, the outer-membrane protein profile showed a time-dependent increase in the intensity of a protein band corresponding to 40 kDa with a concomitant increase in [^{14}C]glucose-binding activity (Fig. 1a). A similar pattern was observed when preinoculum was grown on succinate (Fig. 1b) or benzyl alcohol (data not shown). In the presence of rifampicin (Fig. 1c and d, 10 μg rifampicin ml^{-1} did not affect the cell viability), the induction of the 40 kDa protein in glucose-grown cells was delayed until 36 h, suggesting that the regulation is probably at the level of transcription. Proteoliposomes reconstituted with proteins extracted from the outer membrane of glucose-grown cells showed rapid swelling

in the presence of isosmotic concentration of glucose. However, proteoliposomes reconstituted with outer-membrane proteins from cells grown on naphthalene, benzyl alcohol or succinate did not show any appreciable swelling in the presence of glucose (Table 1).

When grown on a double carbon source (naphthalene + glucose) the intensity of the 40 kDa protein band was lowest during the first exponential phase (up to 7 h) where cells were utilizing naphthalene (Fig. 2a). As the culture entered the second exponential phase (glucose utilization phase) a significant increase in the intensity of the 40 kDa protein was observed (12–18 h, Fig. 2a). These observations were supported by a concomitant increase in the [^{14}C]glucose-binding activity during the second exponential phase of the growth (Fig. 2c). Similar protein profiles and [^{14}C]glucose-binding activity trends were observed for succinate + glucose (Fig. 2b and d) and benzyl alcohol + glucose (data not shown).

2D Gel electrophoresis and identification of the 40 kDa protein

The 2D gel electrophoretic profiles of outer-membrane proteins extracted from CSV86 cells grown on glucose, naphthalene or naphthalene + glucose are shown in Fig. 3. Glucose-grown cells showed a prominent protein spot of 40 kDa (Fig. 3a, circled) which was absent in the naphthalene-grown cells (Fig. 3b). However, on a double carbon source such as naphthalene + glucose, the protein level increased progressively (Fig. 3c and d, circled) and cells started utilizing glucose. The LC-MS/MS analysis of

Table 1. Liposome swelling assay using total outer-membrane protein extracted from *P. putida* CSV86 grown on different carbon sources

Specific activity represents the measurement using glucose as solute.

Cells grown on:	Specific activity (ΔA_{500} min^{-1} mg^{-1})
Glucose	2.0
Naphthalene	0.12
Benzyl alcohol	0.34
Succinate	0.18

tryptic digests of the prominent protein spot corresponding to the 40 kDa protein from glucose-grown cells is summarized in Table 2. Internal peptide fragments obtained from tryptic digestion were subjected to LC-MS/MS analysis and the peptide sequences obtained showed close identity and similarity with Porin B of various *Pseudomonas* species (Table 2). Further, BLAST analysis using one of the longest internal peptides (30mer, query sequence) obtained from the 40 kDa protein showed significant identity and similarity with annotated OprB/Porin B from various *Pseudomonas* species (Table 3).

Purification and properties of the 40 kDa glucose-inducible protein

The 40 kDa glucose-inducible outer-membrane protein was purified to apparent homogeneity (Fig. 4a). The native molecular mass was determined to be 125 kDa by gel

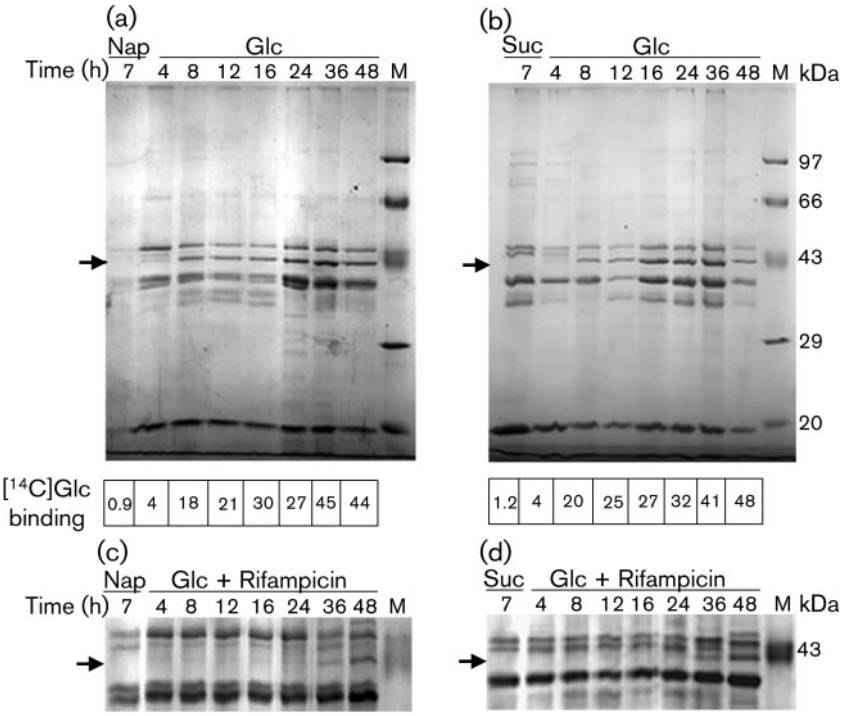

Fig. 1. Transcriptional activation of a 40 kDa outer-membrane protein by glucose (Glc) in *P. putida* CSV86. Cultures grown for the indicated times on naphthalene (Nap; a, c) and succinate (Suc; b, d) were used as inoculum. In (c) and (d), rifampicin (10 μg ml^{-1}) was incorporated in the medium. Treatment of cells with 10 μg rifampicin ml^{-1} for 48 h did not affect the viability of cells and the c.f.u. count at the end of 48 h was 3.5×10^9 cells ml^{-1} for treated cells and 2×10^9 cells ml^{-1} for untreated cells. Protein was extracted as described in Methods and 10 μg was loaded onto each lane. Lane M, standard molecular mass (kDa) proteins: phosphorylase b (97), BSA (66), ovalbumin (43), carbonic anhydrase (29) and soyabean trypsin inhibitor (20). Arrowheads indicate glucose-inducible outer-membrane protein (40 kDa). [^{14}C]Glucose-binding activity of the outer-membrane protein [pmol (mg protein)$^{-1}$] is given in (a) and (b). Images in (c) and (d) are cropped to show only the relevant portion of the gels.

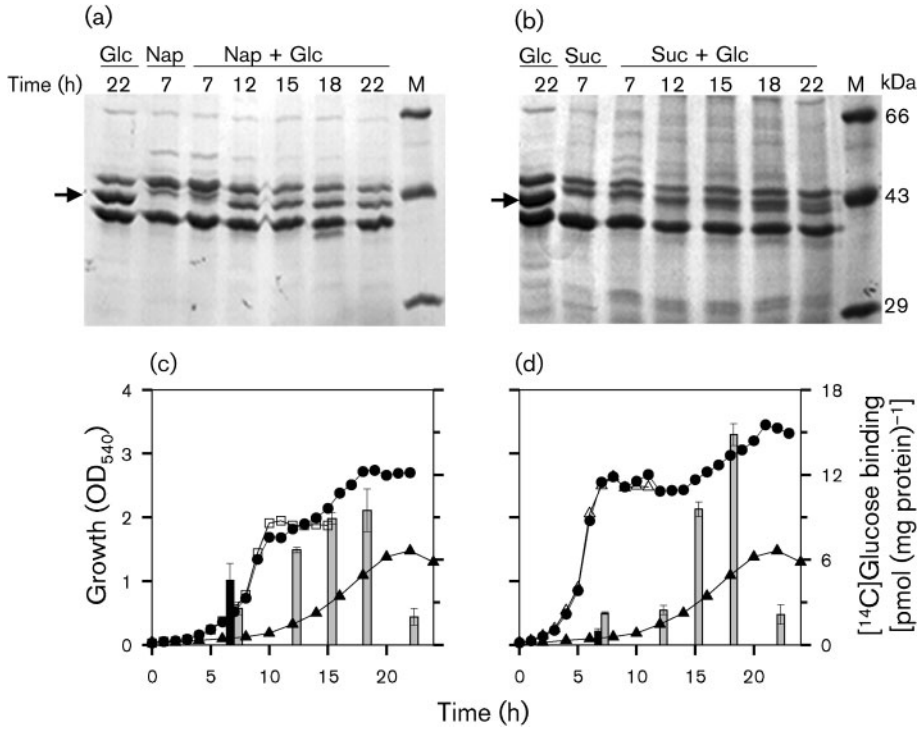

Fig. 2. Growth-dependent induction and activity profile of 40 kDa outer-membrane protein in *P. putida* CSV86 grown on double carbon sources. (a, b) Cells were grown on napthalene + glucose (a) or succinate + glucose (b) for the durations indicated. Each lane contained 10 μg protein. Lane M, standard molecular mass markers. Arrowheads denote the glucose-inducible outer-membrane protein (40 kDa). (c, d) [^{14}C]Glucose-binding activity of the outer-membrane protein obtained from CSV86 cells grown either on napthalene (c, black bar), succinate (d, black bar) or a double carbon source (c, d, grey bars). Growth profiles on naphthalene (□), succinate (△), glucose (▲) and double carbon sources (●) are shown. Error bars, SD.

filtration on Sephacryl S-200HR column under native conditions (Fig. 4b). SDS-PAGE revealed a subunit molecular mass of 40 kDa (Fig. 4a), suggesting that the native protein was a homotrimer. Treatment of the purified protein in the presence or absence of β-mercaptoethanol in a boiling water bath (~95 °C) for 10 min showed relatively lower electrophoretic mobility on SDS-PAGE compared with the protein treated at 25 °C (Fig. 4c).

Functional characterization

The purified 40 kDa protein inserted spontaneously into lipid bilayers and formed conducting channels. Distinct

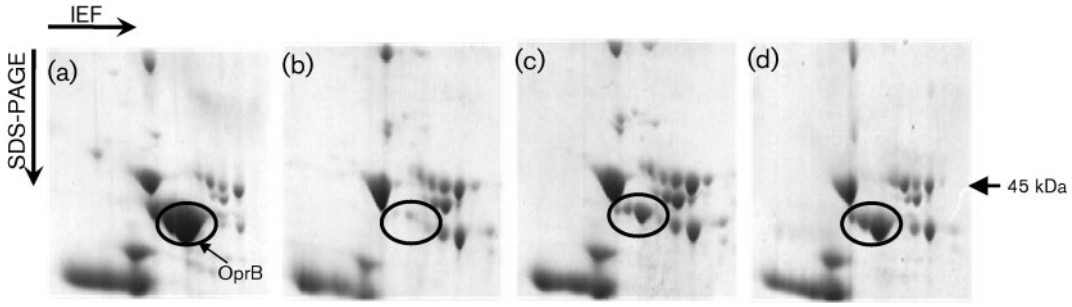

Fig. 3. 2D Gel electrophoresis of the outer-membrane protein fraction of *P. putida* CSV86. Cells were grown on glucose (a) or naphthalene (b) or naphthalene + glucose (c, d) for 7 h (c) or 18 h (d). In the first dimension, the isoelectric focusing was performed using ampholine (pH range, 3.5–10) for 10 000 V h. In the second dimension, discontinuous SDS-PAGE (12 %) was used. About 40 μg outer-membrane protein fractions was used per gel. The gels were stained overnight with Coomassie Brilliant Blue, destained, dried and visualized. The spot encircled in (a) was excised and analysed further by LC-MS/MS as described in Methods. Proteins circled in (b)–(d) increased in intensity.

Table 2. LC-MS/MS analysis of glucose-inducible outer-membrane protein from *P. putida* CSV86

Outer-membrane protein spots from 2D gel of glucose-grown cells (A samples) and purified protein from SDS-PAGE (B samples) were used. NA, Gene locus not assigned.

Sample	Protein identified*	Organism	Mascot score	Accession no.	
				TIGR	Swiss-Prot
A1	Porin B	*P. putida* KT2440	157	PP1019	Q88P34_PSEPK
A2	Porin B	*P. syringae* pv. *phaseolicola*	83	PSPPH1185	Q48MC0_PSE14
B1	Porin B	*P. putida* KT2440	172	PP1019	Q88P34_PSEPK
B2	OprB precursor	*P. syringae* pv. *phaseolicola*	82	PSYR1117	Q4ZXF1_PSEU2
B3	Putative OprD	*P. fluorescens*	49	NA	Q3LAH7_PSEFL

*Identification based on the obtained annotated genes. Eleven peptide fragments obtained by LC-MS/MS of both samples were: KLSGSGTKG; RIHVNDDVKK; KLAITERS; RLTQMWIKQ; KDDIGFGVARI; KYFDGALDVKF; RLGYYYSTAKA; KILGWHDAEFKL; RAGQFSSVQEVWGRG; KSPGGVDEVDNALVAGLKI; and KYNITPEFFVQVGAFEQNPSNLETGNGFKL. The underlined peptide sequences were common to both samples A and B. Peptide coverage is 28 %.

opening and closing events could be observed (Fig. 4d). Analysis of currents from membranes containing only a single channel each revealed a slope conductance of 166 ± 6 pS in 1 M KCl (Fig. 4d). No subconductance states were observed in any of the recordings (over 6000 single channel events observed). The probability of channel opening was independent of applied voltage from −120 mV to +120 mV (data not shown). None of the recordings (~ 6000 single channel events observed) showed any co-operative or sequential opening or closing of three channels present in the trimeric protein.

Permeation of sugars into liposomes was monitored by swelling of proteoliposomes and normalized to the rate of glucose permeation. Among the monosaccharides tested, only arabinose showed a higher rate of permeation than glucose (Fig. 5a). The rate of fructose permeation was slightly lower than that of glucose. However, the rates of permeation were significantly lower for sucrose (disaccharide) and raffinose (trisaccharide). Proteoliposomes also showed permeation of organic acids, aromatic compounds and amino acids with rates lower than that of glucose (Fig. 5a).

The selectivity of the 40 kDa outer-membrane protein was analysed by chasing the bound [^{14}C]glucose with various unlabelled compounds added in 100-fold molar excess (Fig. 5b). A significant reduction in [^{14}C]glucose-binding was found with unlabelled glucose (90 %) but not with various other sugars (4–12 %), organic acids (0–25 %) or aromatic compounds (4–15 %) (Fig. 5b). A binding isotherm demonstrated saturation above 10 μM and an apparent dissociation constant (K_d) of 1.3 μM. (Fig. 5c). These results demonstrate that the 40 kDa outer-membrane protein has a specific and saturable glucose-binding site.

DISCUSSION

Carbon catabolite repression (CCR) is regarded as one of the major bottlenecks in the success of bioremediation to remove toxic aromatic pollutants from the environment. *P.*

Table 3. BLAST analysis of the 40 kDa outer-membrane protein using the LC-MS/MS peptide fragment as query

The query sequence was KYNITPEFFVQVGAFEQNPSNLETGNGFKL.

Accession no.	Organism	Protein	Identity (%)	Similarity (%)	e-value
167032032	*P. putida* GB-1	Carbohydrate-selective porin OprB	100	100	3.00E-20
170723366	*P. putida* W619	Carbohydrate-selective porin OprB	100	100	3.00E-20
26987755	*P. putida* KT2440	Porin B	100	100	3.00E-20
148546300	*P. putida* F1	Carbohydrate-selective porin OprB	96	96	5.00E-19
104783378	*P. putida* L48	Glucose-selective porin OprB	93	96	3.00E-18
237799896	*P. syringae* pv. *oryzae*	Carbohydrate-selective porin OprB	83	90	4.00E-14
213968357	*P. syringae* pv. *tomato*	Porin B	83	90	4.00E-14
218891735	*P. aeruginosa* LESB58	Putative glucose-sensitive porin	76	93	5.00E-13
15597487	*P. aeruginosa* PAO1	Glucose-sensitive porin	76	93	5.00E-13
77460797	*P. fluorescens* Pf0-1	Carbohydrate-selective porin OprB	73	90	3.00E-11

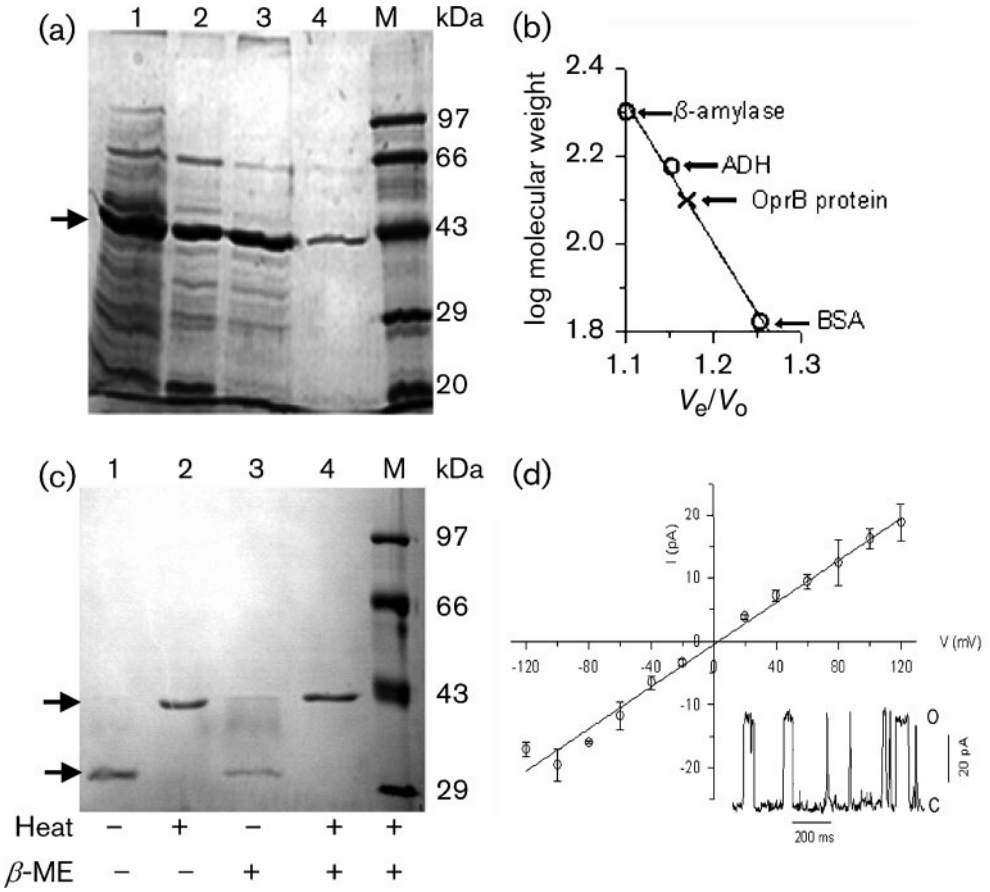

Fig. 4. Purification and properties of the 40 kDa outer-membrane protein of *P. putida* CSV86. (a) Electrophoretic resolution of the purified 40 kDa protein (arrowhead) by SDS-PAGE (10 %). Lane 1, Outer membrane pellet extract; 2, Triton X-100 (0.1 %) supernatant; 3, DEAE-Sephacel pool; 4, Sephacryl-S200-HR pool; M, standard molecular mass proteins. (b) Plot of log molecular mass versus V_e/V_o of purified native OprB protein run on Sephacryl S-200-HR gel filtration column chromatography (×). The standard molecular mass marker proteins (kDa) used were β-amylase (200), alcohol dehydrogenase (150) and BSA (66). (c) Electrophoretic resolution of glucose-inducible 40 kDa outer-membrane protein on SDS-PAGE when treated as follows: lane 1, 25 °C in gel loading buffer [0.2 % (w/v) SDS, 20 % (v/v) glycerol, 125 mM Tris-HCl (pH 6.8)] with no β-mercaptoethanol (β-ME); 2, 100 °C for 10 min in gel loading buffer with no β-ME; 3, 25 °C in gel loading buffer containing 3 % (v/v) β-ME; 4, 100 °C for 10 min in gel loading buffer containing β-ME. Lane M, molecular mass marker (in kDa). Arrowheads denote the change in the mobility of 40 kDa protein. (d) Current–voltage plot of the 40 kDa protein in planar lipid bilayers. Current observed on single channel openings has been plotted against applied potential. The best fit line has a slope of 166 ± 6 pS in 1 M KCl. The inset shows a representative series of channel openings observed at +100 mV. Closed (C) and open (O) states of the channel are indicated.

putida CSV86 preferentially utilizes aromatics or organic acids over glucose and co-metabolizes aromatics and organic acids (Basu *et al.*, 2006). Previously, we have demonstrated that glucose induces glucose-6-phosphate dehydrogenase (Zwf), an intracellular glucose-metabolizing enzyme (Basu & Phale, 2006) and a 43 kDa glucose-binding periplasmic space protein (Basu *et al.*, 2007). Both Zwf and the 43 kDa protein are repressed by aromatics as well as by organic acids (Basu & Phale, 2006; Basu *et al.*, 2007). The present study substantiates and extends these findings by demonstrating that the regulatory event commences at the level of the outer membrane to effectively modulate glucose transport in CSV86.

The close association between the 40 kDa protein and its role in glucose uptake across the outer membrane of CSV86 and the repression by aromatics and organic acid was established by: (i) the high level of a 40 kDa outer-membrane protein and significant [^{14}C]glucose-binding activity from glucose-grown cells compared with aromatic- or organic acid-grown cells; (ii) an increased level of protein and activity in the second exponential phase (glucose utilization phase) when grown on a double carbon source (naphthalene + glucose); and (iii) a high permeation rate for glucose with the outer-membrane protein preparation from glucose-grown cells compared with naphthalene-, benzyl alcohol- or succinate-grown cells.

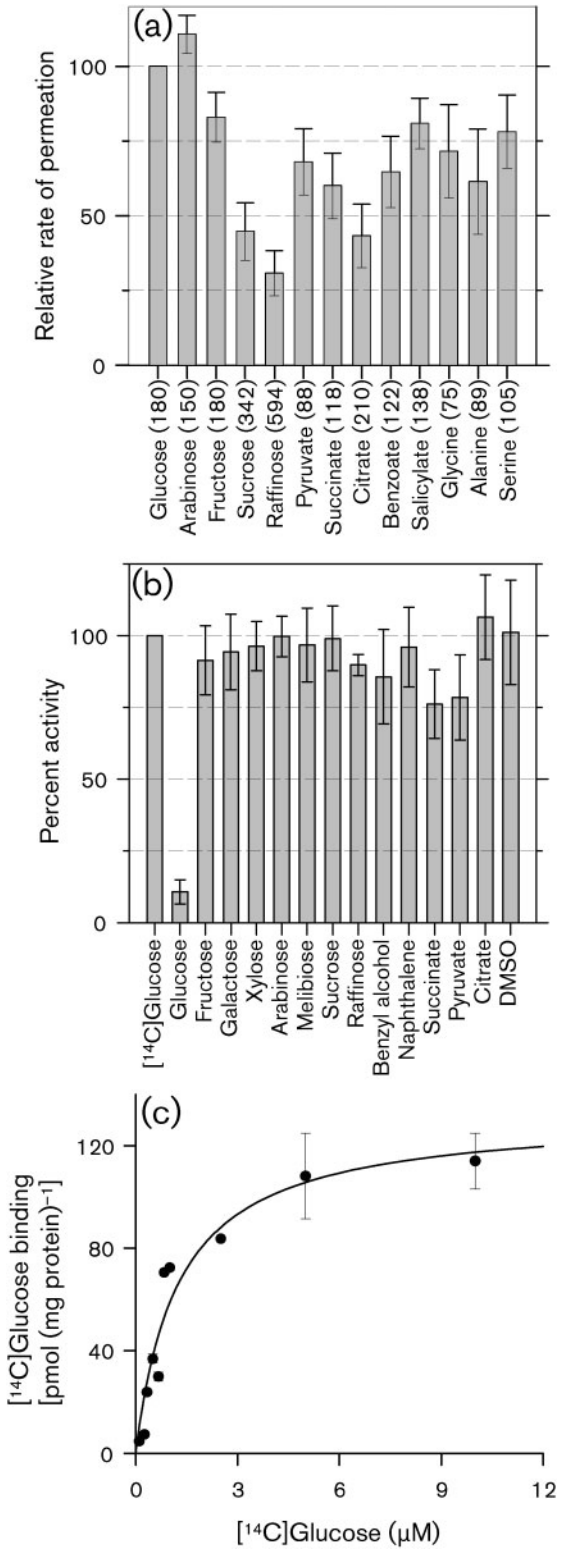

Fig. 5. Permeation properties and specificity of the purified 40 kDa protein of *P. putida* CSV86. (a) Liposome swelling assay. The percentage rate of permeation for solutes was calculated by taking the rate of permeation of glucose as 100 %. The molecular mass is shown in parentheses. (b) Substrate specificity of the 40 kDa protein, as described in Methods (c) Substrate saturation plot of binding of [^{14}C]glucose to the 40 kDa protein versus an increasing concentration of [^{14}C]glucose.

The molecular properties of OprB of CSV86 were found to be comparable to those reported from *P. aeruginosa* and *P. putida* (Saravolac *et al.*, 1991; Wylie *et al.*, 1993). The functional structure of OprB of CSV86 (Fig. 4) appears to be a homotrimer which is similar to that reported for OmpF, LamB of *E. coli* (Cowan *et al.*, 1992; Nikaido, 2003) and OprP of *Pseudomonas aeruginosa* (Hancock & Benz, 1986). The channels were found to be voltage-independent with an average channel conductance of 166 ± 6 pS in 1 M KCl. The observed conductance value is intermediate between the conductance reported for OprB of *P. aeruginosa* (35 pS at 50 mV; Wylie *et al.*, 1993), *P. putida* (25 pS at 20 mV; Saravolac *et al.*, 1991) and OprP of *P. aeruginosa* (250 pS at 50 mV; Hancock & Benz, 1986) under similar experimental conditions. The observed higher conductance for OprB of CSV86 could be due to the opening of all three channels of the trimeric protein, as no subconductance states corresponding to one or two of the constituent channels opening were observed. This is in contrast with OmpF of *E. coli*, where the co-operative opening of the trimer followed by sequential closing of the individual channels was observed (Phale *et al.*, 1997). Although OprB of CSV86 can permeate sugars, organic acids and amino acids through it, the channel showed a specific binding site for glucose with a dissociation constant of 1.3 μM (Fig. 5). The glucose binding site is saturable and the observed affinity of OprB from CSV86 (1.3 μM) was significantly higher than that determined for OprB of *P. putida* (110 mM) and *P. aeruginosa* (380 mM) using macroscopic conductance inhibition experiments (Saravolac *et al.*, 1991; Wylie *et al.*, 1993). Micromolar affinity was reported previously from *P. aeruginosa* for the outer-membrane protein OprP for a binding site of phosphate (Hancock & Brinkman, 2002). The OprB-mediated glucose transport system was functionally characterized from *P. chlororaphis*, *Burkholderia cepacia* and *P. fluorescens* at the whole-cell level. The kinetic studies showed that the system is inducible and had a K_m in the range of 0.3–5 μM for glucose (Adewoye *et al.*, 1998; Hancock & Brinkman, 2002). The presence of a saturable high-affinity glucose-binding site on OprB of CSV86 strongly implicates that it is a part of a high affinity glucose transport system. In *P. aeruginosa*, this transport system is proposed to consist of at least two components (Hancock & Carey, 1980; Wylie *et al.*, 1993) and is reported to have a K_m of 8 μM for glucose compared with that of the low affinity (1–2 mM) transport system (Midgley & Dawes, 1973). The presence of a binding site of such high affinity might also be responsible for slow intake of glucose which in turn causes slow growth of CSV86 on glucose [the strain reaches stationary phase in 22 h (Basu *et al.*, 2006)].

The involvement of OprB in the transport of glucose in *P. aeruginosa* (Wylie *et al.*, 1993; Wylie & Worobec, 1993, 1994, 1995) and *P. putida* (Saravolac *et al.*, 1991), and OprP in the transport of phosphate in *P. aeruginosa* (Hancock *et al.*, 1982; Hancock & Benz, 1986), has been reported. Once the solute is transported across the outer

membrane, solute-specific periplasmic protein can selectively bind to the solute and present it to the ABC transporter system located in the inner membrane for inward transport (Higgins *et al.*, 1990). In *Pseudomonas*, the inner membrane glucose transport component has not yet been identified; however, a specific transport component has been studied in detail for maltose uptake in *E. coli* (Chen *et al.*, 2001). The downregulation of genes involved in the glucose uptake during growth on glucose and toluene was observed in *P. putida* KT2440 which can allow the uptake of glucose via the oxidative and phosphorylative pathway (del Castillo & Ramos, 2007; del Castillo *et al.*, 2008).

The present and previous studies demonstrate that the glucose transport system in *P. putida* CSV86 has a glucose-inducible nature and that it involves an outer membrane channel OprB and periplasmic glucose binding protein. In CSV86, the only mode for glucose uptake is the phosphorylative pathway (Basu & Phale, 2006) which involves glucose transport through membranes. During growth on aromatics plus glucose, aromatics enter the cell and repress the glucose transport components, i.e. the periplasmic glucose-binding protein (Basu *et al.*, 2007) and the outer-membrane protein OprB. This repression could be mediated either directly by aromatics or through organic acids generated as metabolic intermediates during aromatic compound metabolism. Thus, the repression of the phosphorylative pathway at the glucose transport and enzymic level (Zwf; Basu & Phale, 2006) probably relieves CSV86 from glucose-mediated CCR on aromatics and leads to the utilization of aromatic compounds over glucose.

ACKNOWLEDGEMENTS

We thank Professor R. Banerjee and Mr Manas, Department of Biosciences and Bioengineering, IIT-Bombay, for their initial help in liposome preparation. P. S. P. would like to thank the Department of Science and Technology, Government of India for the research grant.

REFERENCES

Adewoye, L. O. & Worobec, E. A. (2000). Identification and characterization of the *gltK* gene encoding a membrane-associated glucose transport protein of *Pseudomonas aeruginosa*. *Gene* **253**, 323–330.

Adewoye, L. O., Tschetter, L., O'Neil, J. & Worobec, E. A. (1998). Channel specificity and secondary structure of the glucose-inducible porins of *Pseudomonas* spp. *J Bioenerg Biomembr* **30**, 257–267.

Alexander, M. (1981). Biodegradation of chemicals of environmental concern. *Science* **211**, 132–138.

Basu, A. & Phale, P. S. (2006). Inducible uptake and metabolism of glucose by the phosphorylative pathway in *Pseudomonas putida* CSV86. *FEMS Microbiol Lett* **259**, 311–316.

Basu, A., Dixit, S. S. & Phale, P. S. (2003). Metabolism of benzyl alcohol via catechol *ortho*-pathway in methylnaphthalene-degrading *Pseudomonas putida* CSV86. *Appl Microbiol Biotechnol* **62**, 579–585.

Basu, A., Apte, S. K. & Phale, P. S. (2006). Preferential utilization of aromatic compounds over glucose by *Pseudomonas putida* CSV86. *Appl Environ Microbiol* **72**, 2226–2230.

Basu, A., Shrivastava, R., Basu, B., Apte, S. K. & Phale, P. S. (2007). Modulation of glucose transport causes preferential utilization of aromatic compounds in *Pseudomonas putida* CSV86. *J Bacteriol* **189**, 7556–7562.

Benz, R., Schmid, A. & Vos-Scheperkeuter, G. H. (1987). Mechanism of sugar transport through the sugar-specific LamB channel of *Escherichia coli* outer membrane. *J Membr Biol* **100**, 21–29.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.

Chen, J., Sharma, S., Quiocho, F. A. & Davidson, A. L. (2001). Trapping the transition state of an ATP-binding cassette transporter: evidence for a concerted mechanism of maltose transport. *Proc Natl Acad Sci U S A* **98**, 1525–1530.

Collier, D. N., Hager, P. W. & Phibbs, P. V., Jr (1996). Catabolite repression control in the Pseudomonads. *Res Microbiol* **147**, 551–561.

Cowan, S. W., Schirmer, T., Rummel, G., Steiert, M., Ghosh, R., Pauptit, R. A., Jansonius, J. N. & Rosenbusch, J. P. (1992). Crystal structures explain functional properties of two *E. coli* porins. *Nature* **358**, 727–733.

Daddaoua, A., Krell, T. & Ramos, J. L. (2009). Regulation of glucose metabolism in *Pseudomonas*: the phosphorylative branch and entner-doudoroff enzymes are regulated by a repressor containing a sugar isomerase domain. *J Biol Chem* **284**, 21360–21368.

del Castillo, T. & Ramos, J. L. (2007). Simultaneous catabolite repression between glucose and toluene metabolism in *Pseudomonas putida* is channeled through different signaling pathways. *J Bacteriol* **189**, 6602–6610.

del Castillo, T., Duque, E. & Ramos, J. L. (2008). A set of activators and repressors control peripheral glucose pathways in *Pseudomonas putida* to yield a common central intermediate. *J Bacteriol* **190**, 2331–2339.

Duetz, W. A., de Jong, C., Williams, P. A. & van Andel, J. G. (1994). Competition in chemostat culture between *Pseudomonas* strains that use different pathways for the degradation of toluene. *Appl Environ Microbiol* **60**, 2858–2863.

Haigler, B. E., Pettigrew, C. A. & Spain, J. C. (1992). Biodegradation of mixtures of substituted benzenes by *Pseudomonas* sp. strain JS150. *Appl Environ Microbiol* **58**, 2237–2244.

Hancock, R. E. & Benz, R. (1986). Demonstration and chemical modification of a specific phosphate binding site in the phosphate-starvation-inducible outer membrane porin protein P of *Pseudomonas aeruginosa*. *Biochim Biophys Acta* **860**, 699–707.

Hancock, R. E. & Brinkman, F. S. (2002). Function of *Pseudomonas* porins in uptake and efflux. *Annu Rev Microbiol* **56**, 17–38.

Hancock, R. E. & Carey, A. M. (1980). Protein D1 – a glucose-inducible, pore-forming protein from the outer membrane of *Pseudomonas aeruginosa*. *FEMS Microbiol Lett* **8**, 105–109.

Hancock, R. E., Poole, K. & Benz, R. (1982). Outer membrane protein P of *Pseudomonas aeruginosa*: regulation by phosphate deficiency and formation of small anion-specific channels in lipid bilayer membranes. *J Bacteriol* **150**, 730–738.

Higgins, S. J. & Mandelstam, J. (1972). Regulation of pathways degrading aromatic substrates in *Pseudomonas putida*. Enzymic response to binary mixtures of substrates. *Biochem J* **126**, 901–916.

Higgins, C. F., Hyde, S. C., Mimmack, M. M., Gileadi, U., Gill, D. R. & Gallagher, M. P. (1990). Binding protein-dependent transport systems. *J Bioenerg Biomembr* **22**, 571–592.

Holtel, A., Marqués, S., Möhler, I., Jakubzik, U. & Timmis, K. N. (1994). Carbon source-dependent inhibition of *xyl* operon expression of the *Pseudomonas putida* TOL plasmid. *J Bacteriol* **176**, 1773–1776.

Joshi, B., Schmid, R., Altendorf, K. & Apte, S. K. (2004). Protein recycling is a major component of post-irradiation recovery in *Deinococcus radiodurans* strain R1. *Biochem Biophys Res Commun* **320**, 1112–1117.

Kim, J., Yeom, J., Jeon, C. O. & Park, W. (2009). Intracellular 2-keto-3-deoxy-6-phosphogluconate is the signal for carbon catabolite repression of phenylacetic acid metabolism in *Pseudomonas putida* KT2440. *Microbiology* **155**, 2420–2428.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Mahajan, M. C., Phale, P. S. & Vaidyanathan, C. S. (1994). Evidence for the involvement of multiple pathways in the biodegradation of 1- and 2-methylnaphthalene by *Pseudomonas putida* CSV86. *Arch Microbiol* **161**, 425–433.

McFall, S. M., Abraham, B., Narsolis, C. G. & Chakrabarty, A. M. (1997). A tricarboxylic acid cycle intermediate regulating transcription of a chloroaromatic biodegradative pathway: fumarate-mediated repression of the *clcABD* operon. *J Bacteriol* **179**, 6729–6735.

Midgley, M. & Dawes, E. A. (1973). The regulation of transport of glucose and methyl alpha-glucoside in *Pseudomonas aeruginosa*. *Biochem J* **132**, 141–154.

Montal, M. & Mueller, P. (1972). Formation of bimolecular membranes from lipid monolayers and a study of their electrical properties. *Proc Natl Acad Sci U S A* **69**, 3561–3566.

Müller, C., Petruschka, L., Cuypers, H., Burchhardt, G. & Herrmann, H. (1996). Carbon catabolite repression of phenol degradation in *Pseudomonas putida* is mediated by the inhibition of the activator protein PhlR. *J Bacteriol* **178**, 2030–2036.

Nakajima, A., Hoshikawa, M. & Nakae, T. (1998). Antibiotic stress induces a large amount of outer membrane protein in *Pseudomonas aeruginosa*. *FEMS Microbiol Lett* **165**, 261–265.

Nestorovich, E. M., Sugawara, E., Nikaido, H. & Bezrukov, S. M. (2006). *Pseudomonas aeruginosa* porin OprF: properties of the channel. *J Biol Chem* **281**, 16230–16237.

Ng, F. M. & Dawes, E. A. (1973). Chemostat studies on the regulation of glucose metabolism in *Pseudomonas aeruginosa* by citrate. *Biochem J* **132**, 129–140.

Nikaido, H. (2003). Molecular basis of bacterial outer membrane permeability revisited. *Microbiol Mol Biol Rev* **67**, 593–656.

Nikaido, H. & Rosenberg, E. Y. (1983). Porin channels in *Escherichia coli*: studies with liposomes reconstituted from purified proteins. *J Bacteriol* **153**, 241–252.

O'Farrell, P. H. (1975). High resolution two-dimensional electrophoresis of proteins. *J Biol Chem* **250**, 4007–4021.

Phale, P. S., Schirmer, T., Prilipov, A., Lou, K. L., Hardmeyer, A. & Rosenbusch, J. P. (1997). Voltage gating of *Escherichia coli* porin channels: role of the constriction loop. *Proc Natl Acad Sci U S A* **94**, 6741–6745.

Saravolac, E. G., Taylor, N. F., Benz, R. & Hancock, R. E. (1991). Purification of glucose-inducible outer membrane protein OprB of *Pseudomonas putida* and reconstitution of glucose-specific pores. *J Bacteriol* **173**, 4970–4976.

Schleissner, C., Olivera, E. R., Fernández-Valverde, M. & Luengo, J. M. (1994). Aerobic catabolism of phenylacetic acid in *Pseudomonas putida* U: biochemical characterization of a specific phenylacetic acid transport system and formal demonstration that phenylacetyl-coenzyme A is a catabolic intermediate. *J Bacteriol* **176**, 7667–7676.

Sly, L. M., Worobec, E. A., Perkins, R. E. & Phibbs, P. V., Jr (1993). Reconstitution of glucose uptake and chemotaxis in *Pseudomonas aeruginosa* glucose transport defective mutants. *Can J Microbiol* **39**, 1079–1083.

Stinson, M. W., Cohen, M. A. & Merrick, J. M. (1977). Purification and properties of the periplasmic glucose-binding protein of *Pseudomonas aeruginosa*. *J Bacteriol* **131**, 672–681.

Stülke, J. & Hillen, W. (1999). Carbon catabolite repression in bacteria. *Curr Opin Microbiol* **2**, 195–201.

Sugawara, E., Nestorovich, E. M., Bezrukov, S. M. & Nikaido, H. (2006). *Pseudomonas aeruginosa* porin OprF exists in two different conformations. *J Biol Chem* **281**, 16220–16229.

Sundara Baalaji, N., Mathew, M. K. & Krishnaswamy, S. (2006). Functional assay of *Salmonella typhi* OmpC using reconstituted large unilamellar vesicles: a general method for characterization of outer membrane proteins. *Biochimie* **88**, 1419–1424.

Tiwari, N. P. & Campbell, J. J. (1969). Enzymatic control of the metabolic activity of *Pseudomonas aeruginosa* grown in glucose or succinate media. *Biochim Biophys Acta* **192**, 395–401.

Trias, J. & Nikaido, H. (1990). Protein D2 channel of the *Pseudomonas aeruginosa* outer membrane has a binding site for basic amino acids and peptides. *J Biol Chem* **265**, 15680–15684.

Trias, J., Rosenberg, E. Y. & Nikaido, H. (1988). Specificity of the glucose channel formed by protein D1 of *Pseudomonas aeruginosa*. *Biochim Biophys Acta* **938**, 493–496.

Wylie, J. L. & Worobec, E. A. (1993). Substrate specificity of the high-affinity glucose transport system of *Pseudomonas aeruginosa*. *Can J Microbiol* **39**, 722–725.

Wylie, J. L. & Worobec, E. A. (1994). Cloning and nucleotide sequence of the *Pseudomonas aeruginosa* glucose-selective OprB porin gene and distribution of OprB within the family *Pseudomonadaceae*. *Eur J Biochem* **220**, 505–512.

Wylie, J. L. & Worobec, E. A. (1995). The OprB porin plays a central role in carbohydrate uptake in *Pseudomonas aeruginosa*. *J Bacteriol* **177**, 3021–3026.

Wylie, J. L., Bernegger-Egli, C., O'Neil, J. D. & Worobec, E. A. (1993). Biophysical characterization of OprB, a glucose-inducible porin of *Pseudomonas aeruginosa*. *J Bioenerg Biomembr* **25**, 547–556.

Edited by: P. Cornelis