Analysis of the *Pseudomonas putida* CA-3 proteome during growth on styrene under nitrogen-limiting and non-limiting conditions

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Pseudomonas putida CA-3 is a styrene-degrading bacterium capable of accumulating medium-chain-length polyhydroxyalkanoate (mclPHA) when exposed to limiting concentrations of a nitrogen source in the growth medium. Using shotgun proteomics we analysed global proteome expression in *P. putida* CA-3 supplied with styrene as the sole carbon and energy source under N-limiting (condition permissive for mclPHA synthesis) and non-limiting (condition non-permissive for mclPHA accumulation) growth conditions in order to provide insight into the molecular response of *P. putida* CA-3 to limitation of nitrogen when grown on styrene. A total of 1761 proteins were identified with high confidence and the detected proteins could be assigned to functional groups including styrene degradation, energy, nucleotide metabolism, protein synthesis, transport, stress response and motility. Proteins involved in the upper and lower styrene degradation pathway were expressed throughout the 48 h growth period under both nitrogen limitation and excess. Proteins involved in polyhydroxyalkanoate (PHA) biosynthesis, nitrogen assimilation and amino acid transport, and outer membrane proteins were upregulated under nitrogen limitation. PHA accumulation and biosynthesis were only expressed under nitrogen limitation. Nitrogen assimilation proteins were detected on average at twofold higher amounts under nitrogen limitation. Expression of the branched-chain amino acid ABC transporter was up to 16-fold higher under nitrogen-limiting conditions. Branched chain amino acid uptake by nitrogen-limited cultures was also higher than that by non-limited cultures. Outer membrane lipoproteins were expressed at twofold higher levels under nitrogen limitation. This was confirmed by Western blotting (immunochemical detection) of cells grown under nitrogen limitation. Our study provides the first global description of protein expression changes during growth of any organism on styrene and accumulating mclPHA (nitrogen-limited growth).

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INTRODUCTION

Pseudomonas putida CA-3 has been reported to utilize styrene as a sole source of carbon and energy and to accumulate the biodegradable polymer medium-chain-length polyhydroxyalkanoate (mclPHA) when nitrogen (N) becomes limited in the growth medium (O'Connor *et al.*, 1996; Ward *et al.*, 2005). These two abilities have been employed in the two-step chemo-biotechnological

conversion of polystyrene, a non-biodegradable polymer, to mclPHA at laboratory scale (Ward *et al.*, 2006). The process has been improved through the controlled feeding of N to the growth medium, which results in a twofold increase in biomass and a 1.4-fold increase in mclPHA accumulation (Goff *et al.*, 2007).

Molecular investigations of the styrene degradation pathway in this organism have been reported (Mooney *et al.*, 2006b; O'Connor *et al.*, 2001). Styrene metabolism in *P. putida* CA-3 proceeds via initial side chain oxidation and involves an upper pathway converting styrene to phenylacetic acid (PA) (O'Connor *et al.*, 1995), and a lower pathway initiated via activation of PA to phenylacetyl-CoA. The further metabolism of phenylacetyl-CoA involves oxidation of the aromatic nucleus, followed by ring cleavage and oxidation of the alicyclic compound,

Abbreviations: CDW, cell dry weight; mclPHA, medium-chain-length polyhydroxyalkanoate; nano-LC MS/MS, nano-electrospray liquid chromatography MS; OMP, outer membrane protein; PA, phenylacetic acid; PHA, polyhydroxyalkanoate; PHB, polyhydroxybutyrate.

Two supplementary tables, listing proteins and peptides identified in the study and spectral count abundance measurements, are available with the online version of this paper.

eventually yielding TCA cycle intermediates (Martinez-Blanco *et al.*, 1990; Mooney *et al.*, 2006b; O'Leary *et al.*, 2002b; Olivera *et al.*, 1998). The *pha* operon in *P. putida* CA-3 consists of two class II MCL-PHA synthases (*phaC1* and *phaC2*) flanking the PHA depolymerase-encoding *phaZ* gene (O'Leary *et al.*, 2005).

While traditional metabolic studies have provided information on single or a small number of molecular targets, high-throughput experimental technologies such as proteomics can generate comprehensive datasets that facilitate understanding of the wider metabolic network of the organism (Park & Lee, 2005). Such system-level analyses are currently changing the strategy of engineering. Bacterial proteome profiling enables elucidation of metabolic networks in bacteria grown under relevant conditions (Krayl *et al.*, 2003; VerBerkmoes *et al.*, 2006). We performed this study with the view to gaining a broader and deeper insight into the response of *P. putida* CA-3 to N limitation when grown on styrene. We compared the proteome of *P. putida* CA-3 grown on styrene under mclPHA accumulating and non-accumulating conditions, expecting that the broad overviews of the protein expression patterns will facilitate identification of proteins and/or pathways possibly affecting styrene metabolism and PHA accumulation. A number of tests were performed with whole cells and extracts of bacterial cells to confirm certain observations arising from the proteomic data and to quantify those responses biochemically.

METHODS

***P. putida* CA-3 growth.** *P. putida* CA-3 (NCIMB 41162) had been isolated previously from a bioreactor containing styrene (O'Connor *et al.*, 1995). Cultures were grown in a 5 l stirred tank reactor (Electrolab) with the styrene supplied continuously as a vapour, as described previously (Goff *et al.*, 2007). The growth medium used was minimal mineral salts (MSM) containing, per litre: 9 g Na₂HPO₄·12H₂O, 1.5 g KH₂PO₄, 0.2 g MgSO₄·7H₂O, 0.002 g CaCl₂ and 1 ml trace element solution (Schlegel *et al.*, 1961). The N source was NH₄Cl (Sigma-Aldrich). For mclPHA accumulation, N was limited with a starting concentration of 65 mg l⁻¹ and a feeding rate of 1.5 mg l⁻¹ h⁻¹ (Goff *et al.*, 2007). To prevent mclPHA accumulation, N was supplied at an initial concentration of 525 mg l⁻¹ and maintained above 400 mg l⁻¹ using a N feeding rate of 45 mg l⁻¹ h⁻¹. Fermentations were performed over a 48 h period at 30 °C, at an agitation rate of 500 r.p.m. and a controlled pH of 6.8. Samples (40 ml) were taken for analysis at 6, 20, 30 and 48 h (T6, T20, T30 and T48, respectively). From each sample, 10 ml was used for protein preparation, another 10 ml was used for the determination of amino acid uptake rate, and the remainder (20 ml) for mclPHA analysis.

Cell growth was monitored spectrophotometrically (OD₅₄₀; Unicam Helios δ UV/VIS spectrophotometer) and cell dry weight (CDW) was obtained via a calibration curve of OD₅₄₀ versus dry weight, where cell suspensions with optical densities ranging from 0.1 to 0.8 were filtered through previously weighed glass fibre membranes (Whatman) and dried at 90 °C until a constant weight was achieved.

Protein sample preparation. Cells from 10 ml culture aliquots taken from the fermenter at different time points over the 48 h period of incubation were collected by centrifugation (5000 g for 10 min at

4 °C) and washed twice with an equal volume of phosphate buffer (50 mM, pH 7). Cells were then resuspended in phosphate buffer to an equal concentration of CDW of 1.2 g l⁻¹ and centrifuged again. Cell pellets were then resuspended in 500 µl BugBuster (primary amine-free, Novagen). Benzonase nuclease (Novagen) and PMSF (Sigma) were added to the suspension at 2 U ml⁻¹ and 0.3 mg ml⁻¹, respectively. Cell suspensions were incubated at 30 °C with gentle shaking (50 r.p.m.) and the cell debris and unbroken cells removed by centrifugation (5000 g for 10 min at 4 °C). The protein concentration of the supernatant was determined by the Lowry method using BSA as a standard. Protein samples (30 µl) were resuspended in double the volume of 2× non-reduced SDS-PAGE sample buffer (80 mM Tris/HCl, pH 6.8, 10% β-mercaptoethanol, 2% SDS, 10%, v/v, glycerol, 0.1% Bromophenol Blue) and heated at 100 °C for 5 min. Denatured proteins were separated on a 10% (w/v) polyacrylamide SDS gel and stained with Coomassie Brilliant Blue (Sigma-Aldrich).

Mass spectrometric peptide analysis. SDS-PAGE gels were cut into bands (16 bands per time point) and the proteins digested in-gel with trypsin according to the method of Shevchenko *et al.* (1996). The resulting peptide mixtures were resuspended in 1% formic acid and analysed by nano-electrospray liquid chromatography MS (nano-LC MS/MS). An HPLC instrument (Dionex) was interfaced with an LTQ ion trap mass spectrometer (ThermoFinnigan). Chromatography buffer solutions (Buffer A, 5% acetonitrile and 0.1% formic acid; Buffer B, 80% acetonitrile and 0.1% formic acid) were used to deliver a 60 min gradient (35 min to 45% Buffer B, 10 min to 90%, hold 10 min, 3 min to 5%, hold for 15 min). A flow rate of 2 µl min⁻¹ was used at the electrospray source. Spectra were searched using the Sequest algorithm (Craig & Beavis, 2004) against the UniProt database restricted to *P. putida* entries (downloaded January 7, 2007). Proteins with (a) a Peptide Prophet probability score greater than 0.99 (Keller *et al.*, 2002), and (b) identified by a minimum of two different peptide spectra were automatically accepted, while spectra for the minority of proteins identified by single spectra were manually checked for quality. Spectral counts were automatically calculated for each protein across different fractions using in-house scripts.

mclPHA extraction and analysis. Cells collected at different time points of fermentation were harvested by centrifugation (5000 g for 10 min at 4 °C) and washed twice with an equal volume of phosphate buffer (50 mM, pH 7). Cells were centrifuged again (5000 g for 10 min at 4 °C) and freeze-dried. The polymer content was determined by subjecting 5–10 mg lyophilized whole cells to acidic methanolysis according to published protocols (Brandl *et al.*, 1988; Lageveen *et al.*, 1988). This method degrades the intracellular PHA to methyl esters of its constituent 3-hydroxyalkanoic acids. The 3-hydroxyalkanoic acid methyl esters were assayed by GC using a Hewlett Packard HP6890 chromatograph equipped with a BP-20 capillary column (30 m by 0.25 mm, 0.25 µm film thickness; J & W Scientific) and a flame-ionization detector (FID). A temperature programme of 60 °C for 3 min, temperature ramp of 5 °C per min, 200 °C for 1 min was used. For the peak identification, methyl esters of 3-hydroxyalkanoic acid were prepared in a similar manner and PHA standards from *P. putida* CA-3 were used (Ward *et al.*, 2005).

Nitrogen assay. The nitrogen concentration in the growth medium was determined by the indophenol method, as described by Scheiner (1976).

Phenylacetyl-CoA ligase enzyme assay. The phenylacetyl-CoA ligase activity was determined by measuring the rate of formation of phenylacetylhydroxamate in the presence of cell lysates (200 µl; prepared as described in the protein sample preparation section), ATP, CoA, PA and neutral hydroxylamine, as previously described (Martinez-Blanco *et al.*, 1990; Ward & O'Connor, 2005). Phenylacetyl-

CoA ligase activities were measured at 30 °C over a 30 min period. The molar extinction coefficient of phenylacetylhydroxamate in the presence of ferric chloride under these conditions is 0.9 mM cm⁻¹ (Martinez-Blanco *et al.*, 1990; Ward & O'Connor, 2005).

Branched chain amino acid uptake. For the measurement of leucine and valine uptake rates, 10 ml samples of cells taken from the fermenter at various time points were harvested by centrifugation (5000 g for 10 min at 4 °C), washed and resuspended in deionized H₂O to OD₅₄₀ 4. Cell suspensions (2 ml) were then supplemented with 1 mM leucine and/or 1 mM valine and incubated at 30 °C with shaking (200 r.p.m.). Samples (50 µl) were removed every 30 min over a 2 h period. The amino acid detection was based on derivatization with isobutyl chloroformate followed by GC-MS, as described elsewhere (Sobolevsky *et al.*, 2004). Briefly, reaction mixture (50 µl) was mixed well with deionized H₂O (50 µl), isobutanol (30 µl), pyridine (10 µl) and isobutyl chloroformate (30 µl). The derivatives were extracted into chloroform (500 µl) by vigorous shaking and subsequent centrifugation (10 000 g for 1 min). A 1 µl volume of the chloroform layer was injected into the splitless mode onto an HP-5 column (20 m by 0.25 mm, 0.33 µm film thickness; J & W Scientific) using an Agilent 6890 gas chromatograph coupled to a 5973 mass-selective detector. The column was held at 50 °C for 2 min then heated to 300 °C at a rate of 10 °C min⁻¹. Under the above conditions, *N*-isobutoxycarbonyl isobutyl esters of leucine and valine had retention times of 14.37 and 15.12 min, respectively.

Immunoblotting of outer membrane protein (OMP). Crude cell lysates, prepared as described in Methods, Protein sample preparation, and used for the mass peptide analysis were separated by SDS-PAGE (10%, w/v) and transferred to a nitrocellulose membrane (Hybond ECL, Amersham Biosciences). For Western blotting, the membrane was probed with mAb 7.3 [mouse antibodies against *P. putida* KT2440 outer membrane lipoprotein as described by Ramos-Gonzalez *et al.* (1992)]. Peroxidase-conjugated goat anti-mouse immunoglobulins (Merck) were used for the chemiluminescent detection. The blots were developed using Immobilon Western chemiluminescent HRP substrate (Millipore) according to the manufacturer's protocol. Immunoluminescence was detected and evaluated using the chemiluminescence-compatible FluorChem imaging system equipped with AlphaEase FC2 software (Alpha Innotech).

RESULTS AND DISCUSSION

In order to gain an insight into the effect of N limitation on the general cellular response, mclPHA accumulation and styrene metabolism, *P. putida* CA-3 was grown in a bioreactor with styrene as the sole source of carbon and energy under two different growth conditions (limited N and non-limited N). The proteomes were profiled over time (T6, T20, T30 and T48) using nano-LC MS/MS (Washburn *et al.*, 2003). This shotgun technique is designed to sample the proteome at a broad level, and uses spectral counts (the frequency with which each protein is detected in the mass spectrometer) as a semiquantitative measure of differential protein expression levels (Cagney *et al.*, 2005). The high-throughput protein annotation and metabolic pathway prediction done in this study were enabled by the availability of the genome sequence of *P. putida* KT2440 (Nelson *et al.*, 2002). The major observations arising from the proteomic analysis

were then confirmed by assay and analysis of whole cells, cell extracts and cytoplasmic contents.

Growth characteristics of *P. putida* CA-3

P. putida CA-3 was grown in the stirred tank bioreactor as previously described (Goff *et al.*, 2007) using styrene as the sole carbon and energy source over 48 h, during which time biomass, N utilization and PHA production were monitored (Fig. 1). After a 48 h fermentation, during which the nitrogen (supplied as NH₄Cl) concentration was kept above 400 mg l⁻¹, a final biomass of 7.2 g l⁻¹ (total 35.8 g from 5 l culture) was obtained (Fig. 1). No PHA was accumulated under these growth conditions. When the N supply was limited (65 mg N l⁻¹ at the start of fermentation), a final CDW of 6.5 g l⁻¹ was achieved (total 30.3 g CDW). However, this weight consisted of 52% PHA (w/w), thus total biomass was 15 g (a total of 10.2 g of mclPHA was obtained from 5 l culture). Thus, N limitation resulted in a 2.4-fold decrease in biomass yield. During the exponential phase (T6–T25), the growth rates for non-limiting and limiting N growth conditions were 0.37 and 0.11 g CDW l⁻¹ h⁻¹, respectively. Therefore, we expected that the different growth rates and concentrations of N source during fermentation would most likely affect the protein expression pattern (Ferenci, 1999).

Shotgun proteomics of *P. putida* CA-3 grown in a bioreactor on styrene under N limitation (permissive for PHA accumulation) and under N excess (not permissive for PHA accumulation)

P. putida CA-3 cultures were grown as described above and harvested at four time points: 6, 20, 30 and 48 h post-inoculation (Fig. 1). In order to produce an extensive dataset of protein expression, the lysate was fractionated by SDS-PAGE and analysed by LC-MS. Over 75 000 peptide tandem mass spectra were used to identify 9033 unique peptides, in turn generating 1761 high-confidence (≥ 0.99 Protein Prophet score) protein identifications (Supplementary Table S1). This represents a substantial proportion (33%) of the predicted *P. putida* proteome, and reflects the extensive MS analysis at different time points under different growth conditions. Moreover, the proteins exhibited a wide range of annotated biophysical (molecular mass, isoelectric point), biochemical (functional annotations) and structural (domains) properties, suggesting that the analysis was not strongly biased in favour of or against any protein class.

Spectral counts were used as a semiquantitative measure of protein abundance (Cagney *et al.*, 2005; Liu *et al.*, 2004; Washburn *et al.*, 2003). Using this analysis, it was possible to follow the expression of individual proteins in the different samples (Supplementary Table S2, Fig. 2). Comparison of our results with a preliminary experiment using *P. putida* CA-3 cultures suggested that the percentage of proteins detected in two MS analyses of independently

Fig. 1. Growth (biomass, g l⁻¹) of *P. putida* CA-3 in minimal salts medium supplied with styrene in the bioreactor under N-limiting (65 mg N l⁻¹ at T0, ■) and non-limiting growth conditions (>500 mg N l⁻¹ at T0, □). N concentration (mg l⁻¹) was tracked over time under N-limiting (▲) and non-limiting growth conditions (△). PHA (as a percentage of CDW) was accumulated under N-limiting conditions only (grey bars). Samples for proteome analysis were taken at 4, 20, 30 and 48 h post-inoculation, as indicated by arrows.

grown cultures is 70–80 % (data not shown). Analysis of 20 ribosomal proteins, whose expression might be assumed to remain relatively independent of the time points and experimental treatment, showed that all 20 were detected in all fractions (% CV for spectra counts=0.34). Identified proteins were next organized into functional pathway groups (e.g. ‘nitrogen assimilation’, ‘amino acid uptake’) using annotations from the Gene Ontology database (Raghava, 2006). High-throughput protein annotation and metabolic pathway prediction were enabled by the availability of the genome sequence of *P. putida* KT2440 (Nelson *et al.*, 2002). The spectral counts, reflecting the relative abundance of all the proteins engaged in the respective pathways, were then totalled. In this way, potentially up- or downregulated pathways could be observed (Table 1). Changes in the relative abundance of individual proteins or whole pathways are discussed below.

Styrene degradation proteins

P. putida CA-3 is a styrene-degrading bacterium that metabolizes styrene aerobically, employing enzymes organized in two distinct catabolic pathways (O’Leary *et al.*, 2002b). An upper pathway involves oxidation of the styrene side chain, leading to epoxystyrene, which is subsequently isomerized to phenylacetaldehyde and then oxidized to PA. The lower pathway involves the conversion

of the PA through CoA intermediates into aliphatic compounds that can enter the Krebs cycle (Fig. 2a).

The genes encoding the upper pathway are organized on the *styABCD* operon, which is regulated at the transcriptional level by the StySR two-component sensory apparatus in this strain (O’Leary *et al.*, 2001) and other species such as *Pseudomonas fluorescens* ST (Beltrametti *et al.*, 1997) and *Pseudomonas* sp. Y2 (Bartolomé-Martin *et al.*, 2004; Velasco *et al.*, 1998). The genes encoding the lower pathway (PACoA catabolon) are typically organized in five contiguous operons: *paaABCEF*, *paaGHIJK*, *paaLMN*, *paaX* and *paaY* (Luengo *et al.*, 2001). Proteins encoded by these genes can be classified into functional units: a transport system (PaaL and PaaM); a phenylacetyl-CoA-activating enzyme (PaaF); a ring-hydroxylating enzymic complex (PaaGHIJK); a ring-fission protein (PaaN); a β -oxidation system (PaaABCE); and two regulatory proteins, a repressor (PaaX) and a putative regulator (PaaY) (Arias *et al.*, 2008; Olivera *et al.*, 1998).

While the typical PACoA catabolon has been suggested to be present in *P. putida* CA-3 (O’Leary *et al.*, 2005), this is the first time, to our knowledge, that all of the enzymes were detected and their relative amounts observed over time when this strain was grown on styrene (Fig. 2b). All of the enzymes of the upper and lower pathways could be detected throughout the 48 h fermentation under both

Fig. 2. Styrene catabolic pathway of *P. putida* CA-3. (a) Styrene upper and lower catabolic pathways, adapted from Nogales *et al.* (2007) and O'Leary *et al.* (2002b). (b) Proteins and their relative abundance in cells grown on styrene under no nutrient limitation (white bars) and under N limitation (grey bars) in the bioreactor. Enzymes and proteins are: StyAB, styrene monooxygenase; StyC, epoxystyrene isomerase; StyD, phenylacetaldehyde dehydrogenase; PaaF, phenylacetyl-CoA ligase; PaaGHIJK, multicomponent phenylacetyl-CoA oxygenase; PaaN and PaaABCDE, enzymes involved in ring-cleavage- and β -oxidation-like reactions of the aliphatic-CoA intermediates. The consensus nomenclature for the *paa* genes proposed by Luengo *et al.* (2001) is used.

N-limiting and non-limiting conditions. However, the relative amounts of the styrene degradation enzymes ranged from 18–22% under N excess to 11–18% under N-limited conditions with respect to the total proteome. StyA and StyD were the most abundant proteins expressed

over time in the upper pathway. StyA was expressed to higher levels at time 6 h under N limitation but to lower levels after this time point (Fig. 2). This observation is in agreement with our previous finding that the catabolic efficiency of styrene degradation dramatically decreases

Table 1. Proteins upregulated in *P. putida* CA-3 grown on styrene under N limitation

Functional group and protein name	N excess*				N limitation				Accession number†
	T6	T20	T30	T48	T6	T20	T30	T48	
PHA synthesis									
Poly(3-hydroxyalkanoate) polymerase 1	0	0	0	0	0	4	5	8	Q88D25
Poly(3-hydroxyalkanoate) polymerase 2	0	0	0	0	0	3	4	14	Q88D23
PHA granule-associated protein (phasin)	0	0	0	0	1	7	19	25	Q88D21
(R)-3-Hydroxydecanoyl-acp :CoA transacylase	0	0	0	0	0	1	1	1	Q88PHAG
N assimilation									
Arginine deiminase	2	4	4	2	2	14	15	11	Q88P52
Nitrogen regulatory protein P-II	3	8	4	5	2	3	16	13	Q88CE7
Proline iminopeptidase	0	0	2	1	2	3	10	8	Q88D01
Transport									
Amino acid ABC transporter; periplasmic amino acid binding protein	2	4	2	3	7	28	16	14	Q88DO5
Putrescine ABC transporter; periplasmic putrescine binding protein	0	1	1	0	2	4	12	10	Q88CJ9
Branched chain amino acid ABC transporter; periplasmic amino acid binding protein	1	2	0	0	5	14	18	16	Q88DF8
Membrane efflux protein	0	1	1	1	1	2	17	12	Q88DA4
Outer membrane/lipoproteins									
Competence lipoprotein ComL; putative	0	0	1	0	1	5	7	8	Q88Q73
HflC protein	0	0	0	0	1	1	6	4	Q88DD6
Lipoprotein, putative	7	16	21	20	22	29	30	31	Q88PU5
Outer membrane lipoprotein OprI	2	3	6	2	2	6	16	11	Q88KG8
OMP; bacterial surface antigen family	0	2	3	0	2	9	18	19	Q88MH2
Stress related									
Peroxidase/catalase HPI	0	2	1	2	1	13	12	13	Q88GQ0
ATP-dependent clp protease subunit clpx	12	13	10	1	22	15	24	28	Q88KI9
Ferric ion binding									
Bacterioferritin	1	2	1	0	1	6	5	5	Q88NX1
Chemotaxis/motility									
Methyl-accepting chemotaxis transducer	1	4	6	8	32	26	37	35	Q88DD9
Metabolism									
Dihydrolipoyl dehydrogenase	6	2	6	1	6	14	11	11	Q88FB1
Phosphorylase	0	1	1	1	0	7	11	14	Q88CY8

*Numbers refer to spectral counts/peptide score.

†Accession number from UniProtKB/TrEMBL.

under PHA-accumulating (N-limiting) conditions (Ward *et al.*, 2005).

With the exception of PaaE, proteins of the lower pathway were abundant under both N-limiting and non-limiting growth conditions (Fig. 2b). PACoA ligase (PaaF) enzyme activity was detected at similar levels throughout the growth cycle in crude extracts of cells supplied with styrene under N limitation (Fig. 3). The current observation supports an earlier observation that N limitation has little effect on the consumption of PA by whole cells grown on styrene (O'Connor *et al.*, 1996). Under full N growth conditions, extracts of *P. putida* CA-3 cells grown on styrene showed a sharp decrease in both the peptide score for PaaF and PACoA ligase activity at T20 and T48, while the peptide score and enzyme activity at T6 and T30 were

higher (Figs 2 and 3). These sharp fluctuations at specific time points raise the possibility that post-translational control of PaaF is occurring.

While Mooney and co-workers demonstrated that StyE, a membrane-associated enzyme, is involved in the facilitated uptake of styrene in *P. putida* CA-3 (Mooney *et al.*, 2006a), we detected only minor levels of StyE under both N-limiting and non-limiting growth conditions with styrene as the growth substrate (Fig. 2b). These findings suggest that other transporters as well as diffusion play a role in addition to StyE-mediated active transport in the uptake of styrene in *P. putida* CA-3.

Recently, Nogales and co-workers have established that the final product of aerobic PA degradation in *P. putida*

Fig. 3. Phenylacetyl-CoA ligase activities in cell extracts of *P. putida* CA-3 cells grown on styrene (grey bars, PHA-accumulating conditions; white bars, PHA non-accumulating conditions) supplied with PA as substrate (0.25 mM). Data are the average of two independent determinations.

KT2440 is succinyl-CoA (Luengo *et al.*, 2004; Nogales *et al.*, 2007). They also demonstrated, through generation of random insertion mutants and biochemical analysis, that this last step of PA degradation is catalysed by β -ketoadypil-CoA thiolase (PaaE). PaaE was also detected in *P. putida* CA-3 when grown on styrene under both N-limiting and non-limiting growth conditions (Fig. 2b).

The regulatory proteins StyS, StyR, PaaX and PaaY were detected at low levels, while PA specific permease (PaaL) was not observed in the proteome of *P. putida* CA-3 grown on styrene. O'Leary and co-workers observed that the transcription of the *styS* gene (upper pathway regulatory sensor kinase) was growth condition-dependent (O'Leary *et al.*, 2002a). Indeed, we observed higher amounts of StyS present under N-limiting conditions (Supplementary Table S1). Del Peso-Santos and co-workers have demonstrated a role for PaaX as a major regulatory protein in the phenylacetyl-CoA catabolon (del Peso-Santos *et al.*, 2006). They recently revealed that phenylacetyl-CoA binds to PaaX, and inactivates PaaX-mediated repression of both the *paa* genes and the *styABCD* operon in *P. putida* Y2 (del Peso-Santos *et al.*, 2008). In agreement with this, PaaX was the most abundant of the regulators detected at T6 after inoculation under non-limiting N conditions (data not shown).

Proteins upregulated under N limitation

The expression of proteins involved in energy/metabolism, amino acid biosynthesis, DNA/RNA biosynthesis and processing, transcription and translation was quite similar under both N excess and N limited conditions (Supplementary Table S1) in *P. putida* CA-3 over time. Most of the N (NH_4Cl) supplied was consumed within 20 h of inoculation under N limitation. Under N limitation (2 mg N l^{-1}) the biomass (CDW) yield was 2.4 g l^{-1} lower than under N excess conditions (Fig. 1). The lack of any

essential nutrient, including N, causes growth limitation (Ferenci, 1999) and physiological changes, whereby cells attempt to optimize nutrient scavenging and growth. We observed differentially expressed proteins belonging to the pathway groups of N assimilation, amino acid transport and uptake, outer membrane/lipoproteins, PHA synthesis, motility/chemotaxis and iron assimilation (Fig. 4). Major representative proteins for each of these functional groups with their respective spectral counts are given in Table 1. In addition, two stress-related proteins (peroxidase/catalase HPI and ATP-dependent Clp protease) were observed to be slightly upregulated under N limitation (Table 1).

Upregulation of N assimilation

As expected, one of the most strongly affected functional groups under N-limited growth were the nitrogen assimilation proteins (Fig. 4). This group is represented by arginine deiminase, N regulatory protein P-II and proline iminopeptidase (Table 1), but also contains proteins such as N utilization substance protein A, nitrate binding protein NasS, nitrite reductase, nitroreductase and aminotransferases. N assimilation proteins represented 8 % of total detected proteins at 30 h of fermentation, while at the same time point of fermentation when N was supplied in excess 4.4 % of total proteins detected were associated with N assimilation. Arginine deiminase (EC 3.5.3.6) together with proline deaminase (EC 3.4.11.5) was the most abundant protein in this group. Arginine deiminase from *Pseudomonas aeruginosa* (PaADI) catalyses the hydrolysis of arginine to citrulline and ammonium ion, which is the first step of the microbial L-arginine degradation pathway (Lu *et al.*, 2006). Proline iminopeptidase is also a hydrolytic enzyme involved in proteolysis as one of the ways to assimilate N when its supply is limited.

In poor growth conditions, the metabolism of cells is oriented towards an economical use of substrates by utilization of the available metabolites for the synthesis of

Fig. 4. Protein groups of *P. putida* CA-3 upregulated during growth on styrene under N-limiting conditions (grey bars, PHA-accumulating conditions) with respect to no nutrient limitation conditions (white bars, PHA non-accumulating conditions).

proteins and nucleic acids (Lavallee *et al.*, 2005). Here, we observed increased levels of amino acid transport proteins under N-limiting conditions (Table 1). This was confirmed in branched chain amino acid uptake experiments, where samples taken from the bioreactor at T48 grown under N-limiting conditions showed 2.2-fold higher leucine uptake compared with cells cultured under non-limiting growth conditions (Fig. 5). All bacterial cell samples were normalized so that they had the same biomass (g CDW l^{-1}) in the amino acid uptake assay. Cells harvested from the N-limited fermentation at T48 consumed 1.5-fold more leucine than cells harvested at T6 over the 2 h test period (Fig. 5). In all samples tested N limited cells had a higher rate of leucine uptake than cells grown under non-limiting conditions (Fig. 5). Similar results were obtained when the amino acid used was valine (data not shown). These data were in agreement with the amounts of branched chain amino acid transporters detected by MS analysis (Table 1).

Fig. 5. Branched chain amino acid (leucine) uptake rate of *P. putida* CA-3 cells grown on styrene in a bioreactor under N non-limiting conditions (a, open symbols) and N-limiting conditions (b, filled symbols). Samples were taken at 6 h (diamonds), 24 h (circles), 30 h (triangles) and 48 h (squares) from the bioreactor. Data are an average from two independent determinations.

Proteins involved in polyamine (putrescine) metabolism were also upregulated under N-limiting growth conditions (Supplementary Table S1). Polyamines (including putrescine and spermidine) are a group of ubiquitous polycations necessary for cell growth (Lu *et al.*, 2002). In *P. aeruginosa*, putrescine can be synthesized from arginine through the arginine decarboxylase pathway (Patel *et al.*, 2006). Through this pathway, arginine is sequentially converted and channelled into the tricarboxylic acid cycle (Patel *et al.*, 2006). In cells grown under N limitation, the polyamine ABC transporters were particularly abundant, reflecting the cellular mechanisms used to deal with the N limitation. Other transport proteins were also upregulated, providing a range of means for scavenging N-based compounds. Santos *et al.* (2004) suggested that the differential expression of ABC transporters serves as a global signal for response to the concentration and the quality of the carbon source, starvation conditions and toxic stress. N limitation also stimulated upregulation of the proteolytic enzymes (Hsl protease, ATP-dependent La protease and SohB).

mcIPHA synthesis occurs only under N limitation

One of the metabolic adaptations to suboptimal N levels during *P. putida* CA-3 growth on styrene is the accumulation of an intracellular carbon and energy storage material in the form of mcIPHA granules (Ward *et al.*, 2006). This has also been observed in many other micro-organisms as a response to stress imposed on cells during imbalanced growth, when essential nutrients are limited and carbon is in abundance (Hoffmann & Rehm, 2005; Rehm, 2007; Wältermann & Steinbüchel, 2005).

PHA accumulation was observed under N-limiting growth conditions when the N levels reached a concentration of 26 mg l^{-1} (T6). PHA accumulation continued throughout the fermentation, during which time N was kept at low levels ($2\text{--}3 \text{ mg l}^{-1}$) using a feeding strategy described by Goff *et al.* (2007). PHA levels increased 12-fold over 14 h (from T6 to T20), with a further 1.5- and 2.2-fold increase over the following 10 and 28 h, respectively (Fig. 1). No PHA was detected in cells grown with N excess.

PHA biosynthetic proteins were detected in the proteome of *P. putida* CA-3 cells grown under N limitation (Fig. 4). We observed poly(3-hydroxyalkanoate) polymerase 1 (PhaC1), poly(3-hydroxyalkanoate) polymerase 2 (PhaC2), 3-hydroxyacyl-CoA-acyl carrier protein transferase (PhaG), and granule-associated proteins 1 and 2 (Table 1). The PHA biosynthetic enzymes were detected at relatively low levels, while the depolymerase was not detected in proteomes of *P. putida* CA-3 during the course of this study. It has been documented that in PHA-producing organisms the polymerase and depolymerase, as well as phasin enzymes, are associated with PHA granules and form a distinct protein surface network (Pötter *et al.*, 2004; Pötter & Steinbüchel, 2005; Sandoval *et al.*, 2007). PHA granules are synthesized by PHA polymerases that

utilize CoA thioesters of the respective 3-hydroxy fatty acids as substrates. Granules are mobilized (degraded) by PHA depolymerases, and formation of the granules depends on the activity of several granule-associated proteins (phasins).

Lipoproteins, chemotaxis-related proteins and iron ion transport proteins are upregulated under N limitation

In cells accumulating PHA (grown under N-limiting conditions) we observed upregulation of other OMPs, such as lipoproteins and lipoprotein-releasing proteins (Lol proteins), that target and anchor lipoproteins to the periplasmic surface of either the inner or the outer membrane (Miyamoto & Tokuda, 2007; Narita & Tokuda, 2006). It has been shown that nutrient limitation induces the expression of proteins in the outer membrane of bacterial cells such as *Pseudomonas* species under carbon, N and phosphorus limitation (Kragelund & Nybroe, 1994). Lipoproteins have been shown to have emulsifying properties that increase surface area and hence enhance the bioavailability of hydrophobic substrates such as styrene (Kuiper *et al.*, 2004; Whang *et al.*, 2008). They are also known to play a role in the bacterial adaptation to changes in environmental conditions such as osmotic stress (Guyard-Nicodème *et al.*, 2008). Among OMPs, OmpA, G, H, E3, F and I were detected in the current study, but only OmpF and OmpA porins were twofold to fourfold upregulated by N limitation (Table 1, Supplementary Table S1). OmpF is a major membrane protein in *P. aeruginosa*, where it has a non-specific porin function (Hancock & Brinkman, 2002). In fact, Li *et al.* (1995) have demonstrated that an *ompF*[−] mutant of *P. aeruginosa* was toluene-tolerant, and therefore it was proposed that toluene enters the cell through the OmpF channel in wild-type cells. In contrast, Volkers and co-workers found that OmpF is downregulated in the presence of toluene excess in a chemostat-based culture of *P. putida* S12 and proposed that this strain is capable of shutting down this channel upon toluene stress (Volkers *et al.*, 2006). In the same study, OmpH was dramatically upregulated (12-fold), while we observed more moderate (twofold) upregulation of this OMP. The higher levels of OmpA observed in this study are in agreement with the findings of Benndorf and co-workers, who identified OmpA as being upregulated in *P. putida* KT2440 in response to lipophilic herbicides (Benndorf *et al.*, 2006).

Proteomic data were confirmed through Western blotting using a highly specific monoclonal antibody (mAb 7.3) which was previously raised against surface determinants of *P. putida* 2440 (LPS and membrane proteins) (Ramos-Gonzalez *et al.*, 1992). Approximately 1.7-fold higher amounts of outer membrane lipoproteins containing O-antigen were detected in cells grown under N limitation compared with cells grown under N excess at T48, as determined by densitometry (Fig. 6). Interestingly, two

Fig. 6. Outer membrane lipoproteins with O-antigen from styrene-grown *P. putida* CA-3 cells grown in a bioreactor. A 30 µl quantity of the whole-cell lysate from each sample was resolved by SDS-PAGE (10%), immunoblotted and probed with mAb 7.3 antibodies (Ramos-Gonzalez *et al.*, 1992).

bands were observed when mAb 7.3 was used. This suggests that *P. putida* CA-3 has more than one unit of O-antigen in its LPS (Fig. 6). Cowell *et al.* (1999) suggested that the increased adhesion to surfaces exhibited by *P. aeruginosa* grown under N limitation is due to an increase in OMP expression.

Under N-limiting (PHA accumulation) conditions, a number of motility-associated proteins were upregulated (Fig. 4, Table 1). The majority of these proteins (~80%) were chemotaxis-related proteins, including chemotaxis histidine kinase CheA, chemotaxis proteins CheV, Y and Z, and a variety of methyl-accepting chemotaxis transducers. These methyl-accepting chemotaxis transducers are outer membrane receptors that mediate chemotaxis to diverse signals, responding to changes in the concentration of attractants and repellents in the environment by changing swimming behaviour (Adler, 1966). This has been observed in *P. putida* in response to various substances, including naphthalene (Grimm & Harwood, 1997; Harwood *et al.*, 1990; Law & Aitken, 2006). The second group of motility-related proteins that was upregulated were flagellar biosynthetic proteins such as FliA, C, F, E, G, K and N. The importance of chemotaxis and flagellins was identified in a study by Raberg and co-workers, who studied flagellation changes in response to nutrient supply and the state of polyhydroxybutyrate (PHB) production in *Ralstonia eutropha* H16 (Raberg *et al.*, 2008).

We have also identified number of proteins involved in iron ion acquisition and storage that were induced by N-limiting growth conditions (Fig. 4, Table 1). The most differentially expressed was bacterioferritin (Q88NX1), a protein that interacts with siderophores. Ferritins are part of a class of iron binding proteins that reversibly sequester excess iron and can thereby serve to ease iron-related oxidative stress (Smith, 2004). Bacterioferritins whose expression levels vary as a function of environmental and intracellular iron levels have been identified in *P. putida* KT2440 (Chen *et al.*, 2009). Similarly, bacterioferritins are highly expressed in biofilms of *P. aeruginosa* (Patrauchan *et al.*, 2007), but they have not been identified as being upregulated under N-limited growth conditions.

Solvent tolerance response by *P. putida* CA-3 to the presence of styrene

When grown on styrene, *P. putida* CA-3 cells face a paradox. On the one hand, styrene represents a carbon source that can be metabolized to yield carbon and energy for growth. On the other hand, with an octanol/water partition coefficient [$\log(P_{OW})$] of 3, styrene falls into a category of organic solvents extremely toxic to micro-organisms, due to their ability to bind to the cells, disturbing membranes by removing lipids and proteins and causing cell lysis (Ramos *et al.*, 2001; Taylor *et al.*, 2008). It is well documented that solvent-tolerant pseudomonads possess three membrane-associated solvent tolerance mechanisms: (A) active efflux of organic solvents, (B) outer membrane changes and (C) cytoplasmic membrane changes (Volkers *et al.*, 2006).

(A) Active efflux of organic solvents. Several laboratories have identified efflux pumps belonging to the resistance–nodulation–cell division (RND) family as being the most important mechanism of solvent tolerance (Kieboom *et al.*, 1998; Ramos *et al.*, 2001). They are energy-dependent active efflux pumps, which export toxic organic solvents to the external medium. This mechanism has also been identified in *P. putida* KT2440 (Benndorf *et al.*, 2006). These efflux pumps were present throughout the fermentation of *P. putida* CA-3 under both N-limiting and N-excess growth conditions. (e.g. UniProtKB/TrEMBL Q88DA4, homologous to toluene efflux pump). Nevertheless, these proteins were present at considerably higher levels (by a factor of 10) by T30 under N-limiting conditions (Table 1). It has been determined elsewhere that the operation of these efflux pumps seems to be coupled to the proton motive force, that they have dual pumping capacity, and that specific and global regulators control their expression at the transcriptional level (Kieboom *et al.*, 1998; Ramos *et al.*, 2002).

(B) Outer membrane changes. An integral part of the efflux systems described above are OMPs, which represent an outer membrane channel by which the pumped molecule is released into the medium (Kieboom & De Bont, 2001). OMPs associated with the efflux pumps (TolC, OmpJ) were detected in the proteome of *P. putida* CA-3 grown on styrene (Supplementary Table S1). As described above in Results and Discussion, Lipoproteins, chemotaxis-related proteins and iron ion transport proteins upregulated under N limitation, a putative lipoprotein is expressed by cells grown on styrene, but increased outer membrane lipoprotein and bacterial surface antigen expression occurs under N limitation.

(C) Cytoplasmic membrane changes. An immediate response of micro-organisms to harsh environmental conditions (chemical or physical stresses) is the readjustment of cell-membrane fluidity by the alteration of their phospholipid composition. One of the key

elements in this response is the *cis* to *trans* isomerization of unsaturated fatty acids by the Cti isomerase, which causes membrane rigidity (Heipieper *et al.*, 1996; von Wallbrunn *et al.*, 2003). However, in this study, Cti isomerase (which is located in the periplasm) was not detected. In agreement with this finding, Junker and co-workers described a *P. putida* DOT-T1E Cti null mutant that is still able to survive at high toluene concentrations, indicating that although important, *cis/trans* isomerization is not essential for bacterial solvent resistance (Junker & Ramos, 1999).

Given that the Cti fatty acid isomerase was not detected in this study, it would appear that efflux pumps and outer membrane lipoproteins (i.e. Q88MH2, Table 1) are more likely to be involved in the maintenance and integrity of the cell membrane in *P. putida* CA-3 grown on styrene, especially under N-limiting conditions.

Stress-related proteins

A number of proteins involved in detoxification, oxidative stress response and protein folding mechanisms were identified with higher spectral counts in cells grown under N limitation, e.g. peroxidase/catalase HPI and ATP-dependent Clp protease (Table 1). It is thought that the catalase/peroxidase subfamily provides protection under oxidative stress in bacteria (Welinder, 1991). Increased catalase levels have been detected in the proteome of *Pseudomonas* sp. M1 and KT2440 when grown on or in the presence of phenol (Santos *et al.*, 2004, 2007). Multiple stress-related proteins, such as AphC, SodB and other antioxidants, are induced in *P. putida* KT2440 cells in the presence of a range of aromatic substrates (Kim *et al.*, 2006). The presence of stress-related proteins in cells grown on styrene supports the claim that styrene has a toxic effect on bacteria cells (Ramos *et al.*, 2001). Bacteria accumulating PHB have been previously reported to upregulate heat shock (HspA) proteins in response to stress (Kang *et al.*, 2008; Tessmer *et al.*, 2007). The authors propose that HspA can act like a PHA granule-associated protein (phasin) which affects PHA granule coalescence (Tessmer *et al.*, 2007). In the current study, Clp protease (subunit ClpX) was upregulated in *P. putida* CA-3 cells under N limitation (Table 1). Recently, we generated a transposon mutant negatively affected in PHA accumulation disrupted in the *clpA* gene, suggesting that the activity of the ClpP protease is important for PHA accumulation in *P. putida* CA-3 (Goff *et al.*, 2009). While some members of the Clp family are involved in proteolysis regulation, this family also has many attributes of molecular chaperones (Squires & Squires, 1992). ClpXP is involved in DNA damage repair, stationary-phase gene expression and protein quality control (Neuwald *et al.*, 1999), and to date more than 50 proteins, including transcription factors, metabolic enzymes, and proteins involved in the starvation and oxidative stress responses, have been identified as substrates (Flynn *et al.*, 2003). Clp protease in *Bacillus subtilis*

ClpP is thought to play a role during heat shock as well as oxidative and salt stress (Volker *et al.*, 1994), while in *P. fluorescens* (O'Toole & Kolter, 1998), it is linked to biofilm formation, which is a bacterial response to specific environmental triggers/stresses.

Conclusions

A wide variety of proteins are expressed in *P. putida* CA-3 in response to the presence of styrene and limiting concentrations of N in the growth medium. The broad range of these proteins indicates the complex and varied response of this bacterium to the presence of a lipophilic substrate (styrene) and to the decrease in the concentration of an essential inorganic nutrient (N). The internal and surface protein expression response also reveals the multifaceted nature of the physiological response of *P. putida* CA-3 to environmental stimuli. The data generated here will allow us to investigate specific molecular targets to examine the effect of gene disruption on styrene metabolism, N assimilation and mclPHA accumulation by *P. putida* CA-3. The data also form a basis for the design and evaluation of future metabolic engineering efforts, where analysis of multiple pathways and global responses will increase as this field moves towards systems biology methods (Park *et al.*, 2008).

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REFERENCES

- Adler, J. (1966). Chemotaxis in bacteria. *Science* **153**, 708–715.
- Arias, S., Olivera, E. R., Arcos, M., Naharro, G. & Luengo, J. M. (2008). Genetic analyses and molecular characterization of the pathways involved in the conversion of 2-phenylethylamine and 2-phenylethanol into phenylacetic acid in *Pseudomonas putida* U. *Environ Microbiol* **10**, 413–432.
- Bartolomé-Martin, D., Martinez-Garcia, E., Mascaraque, V., Rubio, J., Perera, J. & Alonso, S. (2004). Characterization of a second functional gene cluster for the catabolism of phenylacetic acid in *Pseudomonas* sp. strain Y2. *Gene* **341**, 167–179.
- Beltrametti, F., Marconi, A. M., Bestetti, G., Colombo, C., Galli, E., Ruzzi, M. & Zennaro, E. (1997). Sequencing and functional analysis of styrene catabolism genes from *Pseudomonas fluorescens* ST. *Appl Environ Microbiol* **63**, 2232–2239.
- Benndorf, D., Thiersch, M., Loffhagen, N., Kunath, C. & Harms, H. (2006). *Pseudomonas putida* KT2440 responds specifically to chlorophenoxy herbicides and their initial metabolites. *Proteomics* **6**, 3319–3329.
- Brandl, H., Gross, R. A., Lenz, R. W. & Fuller, R. C. (1988). *Pseudomonas oleovorans* as a source of poly(β -hydroxyalkanoates) for potential applications as biodegradable polyesters. *Appl Environ Microbiol* **54**, 1977–1982.
- Cagney, G., Park, S., Chung, C., Tong, B., O'Dushlaine, C., Shields, D. C. & Emili, A. (2005). Human tissue profiling with multidimensional protein identification technology. *J Proteome Res* **4**, 1757–1767.
- Chen, S., Bleam, W. F. & Hickey, W. J. (2009). Simultaneous analysis of bacterioferritin gene expression and intracellular iron status in *Pseudomonas putida* KT2440 by using a rapid dual luciferase reporter assay. *Appl Environ Microbiol* **75**, 866–868.
- Cowell, B. A., Willcox, M. D. P., Herbert, B. & Schneider, R. P. (1999). Effect of nutrient limitation on adhesion characteristics of *Pseudomonas aeruginosa*. *J Appl Microbiol* **86**, 944–954.
- Craig, R. & Beavis, R. C. (2004). TANDEM: matching proteins with tandem mass spectra. *Bioinformatics* **20**, 1466–1467.
- del Peso-Santos, T., Bartolomé-Martin, D., Fernandez, C., Alonso, S., Garcia, J. L., Diaz, E., Shingler, V. & Perera, J. (2006). Coregulation by phenylacetyl-coenzyme A-responsive PaaX integrates control of the upper and lower pathways for catabolism of styrene by *Pseudomonas* sp. strain Y2. *J Bacteriol* **188**, 4812–4821.
- del Peso-Santos, T., Shingler, V. & Perera, J. (2008). The styrene-responsive StyS/StyR regulation system controls expression of an auxiliary phenylacetyl-coenzyme A ligase: implications for rapid metabolic coupling of the styrene upper- and lower-degradative pathways. *Mol Microbiol* **69**, 317–330.
- Ferenci, T. (1999). Regulation by nutrient limitation. *Curr Opin Microbiol* **2**, 208–213.
- Flynn, J. M., Neher, S. B., Kim, Y. I., Sauer, R. T. & Baker, T. A. (2003). Proteomic discovery of cellular substrates of the ClpXP protease reveals five classes of ClpX-recognition signals. *Mol Cell* **11**, 671–683.
- Goff, M., Ward, P. G. & O'Connor, K. E. (2007). Improvement of the conversion of polystyrene to polyhydroxyalkanoate through the manipulation of the microbial aspect of the process: a nitrogen feeding strategy for bacterial cells in a stirred tank reactor. *J Biotechnol* **132**, 283–286.
- Goff, M., Nikodinovic-Runic, J. & O'Connor, K. (2009). Characterisation of temperature sensitive and lipopolysaccharide over-producing transposon mutants of *Pseudomonas putida* CA-3 affected in PHA accumulation. *FEMS Microbiol Lett* **292**, 297–305.
- Grimm, A. C. & Harwood, C. S. (1997). Chemotaxis of *Pseudomonas* spp. to the polyaromatic hydrocarbon naphthalene. *Appl Environ Microbiol* **63**, 4111–4115.
- Guyard-Nicodème, M., Bazire, A., Hemery, G., Meylheuc, T., Mollé, D., Orange, N., Fito-Boncompagni, L., Feuillol, M., Haras, D. & other authors (2008). Outer membrane modifications of *Pseudomonas fluorescens* MF37 in response to hyperosmolarity. *J Proteome Res* **7**, 1218–1225.
- Hancock, R. E. & Brinkman, F. S. (2002). Function of *Pseudomonas* porins in uptake and efflux. *Annu Rev Microbiol* **56**, 17–38.
- Harwood, C. S., Parales, R. E. & Dispensa, M. (1990). Chemotaxis of *Pseudomonas putida* toward chlorinated benzoates. *Appl Environ Microbiol* **56**, 1501–1503.
- Heipieper, H. J., Meulenbeld, G., van Oirschot, Q. & de Bont, J. A. M. (1996). Effect of environmental factors on the *trans/cis* ratio of unsaturated fatty acids in *Pseudomonas putida* S12. *Appl Environ Microbiol* **62**, 2773–2777.
- Hoffmann, N. & Rehm, B. H. A. (2005). Nitrogen-dependent regulation of medium-chain length polyhydroxyalkanoate biosynthesis genes in pseudomonads. *Biotechnol Lett* **27**, 279–282.
- Junker, F. & Ramos, J. L. (1999). Involvement of the *cis/trans* isomerase Cti in solvent resistance of *Pseudomonas putida* DOT-T1E. *J Bacteriol* **181**, 5693–5700.

- Kang, Z., Wang, Q., Zhang, H. & Qi, Q. (2008). Construction of a stress-induced system in *Escherichia coli* for efficient polyhydroxyalkanoates production. *Appl Microbiol Biotechnol* **79**, 203–208.
- Keller, A., Nesvizhskii, A. I., Kolker, E. & Aebersold, R. (2002). Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. *Anal Chem* **74**, 5383–5392.
- Kieboom, J. & De Bont, J. A. M. (2001). Identification and molecular characterization of an efflux system involved in *Pseudomonas putida* S12 multidrug resistance. *Microbiology* **147**, 43–51.
- Kieboom, J., Dennis, J. J., Zylstra, G. J. & De Bont, J. A. M. (1998). Active efflux of organic solvents by *Pseudomonas putida* S12 is induced by solvents. *J Bacteriol* **180**, 6769–6772.
- Kim, Y. H., Cho, K., Yun, S.-H., Kim, J. Y., Kwon, K.-H., Yoo, J. S. & Kim, S. I. (2006). Analysis of aromatic catabolic pathways in *Pseudomonas putida* KT 2440 using a combined proteomic approach: 2-DE/MS and cleavable isotope-coded affinity tag analysis. *Proteomics* **6**, 1301–1318.
- Kragelund, L. & Nybroe, O. (1994). Culturability and expression of outer membrane proteins during carbon, nitrogen, or phosphorus starvation of *Pseudomonas fluorescens* DF57 and *Pseudomonas putida* DF14. *Appl Environ Microbiol* **60**, 2944–2948.
- Krayl, M., Benndorf, D., Loffhagen, N. & Babel, W. (2003). Use of proteomics and physiological characteristics to elucidate ecotoxic effects of methyl *tert*-butyl ether in *Pseudomonas putida* KT2440. *Proteomics* **3**, 1544–1552.
- Kuiper, I., Lagendijk, E. L., Pickford, R., Derrick, J. P., Lamers, G. E. M., Thomas-Oates, J. E., Lugtenberg, B. J. J. & Bloemberg, G. V. (2004). Characterization of two *Pseudomonas putida* lipopeptide biosurfactants, putisolvin I and II, which inhibit biofilm formation and break down existing biofilms. *Mol Microbiol* **51**, 97–113.
- Lageveen, R. G., Huisman, G. W., Preusting, H., Ketelaar, P., Eggink, G. & Witholt, B. (1988). Formation of polyesters by *Pseudomonas oleovorans*: effect of substrates on formation and composition of poly-(R)-3-hydroxyalkanoates and poly-(R)-3-hydroxyalkenoates. *Appl Environ Microbiol* **54**, 2924–2932.
- Lavallee, B., Lessard, P. & Vanrolleghem, P. A. (2005). Review of prokaryote metabolism in view of modeling microbial adaptation from fast growth to starvation conditions. *J Environ Eng Sci* **4**, 517–532.
- Law, A. M. J. & Aitken, M. D. (2006). The effect of oxygen on chemotaxis to naphthalene by *Pseudomonas putida* G7. *Biotechnol Bioeng* **93**, 457–464.
- Li, L., Komatsu, T., Inoue, A. & Horikoshi, K. (1995). A toluene-tolerant mutant of *Pseudomonas aeruginosa* lacking the outer membrane protein F. *Biosci Biotechnol Biochem* **59**, 2358–2359.
- Liu, H., Sadygov, R. G. & Yates, J. R. I. (2004). A model for random sampling and estimation of relative protein abundance in shotgun proteomics. *Anal Chem* **76**, 4193–4201.
- Lu, C.-D., Itoh, Y., Nakada, Y. & Jiang, Y. (2002). Functional analysis and regulation of the divergent *spuABCDEFHG*-*spuI* operons for polyamine uptake and utilization in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* **184**, 3765–3773.
- Lu, X., Li, L., Wu, R., Feng, X., Li, Z., Yang, H., Wang, C., Guo, H., Galkin, A. & other authors (2006). Kinetic analysis of *Pseudomonas aeruginosa* arginine deiminase mutants and alternate substrates provides insight into structural determinants of function. *Biochemistry* **45**, 1162–1172.
- Luengo, J. M., Garcia, J. L. & Olivera, E. R. (2001). The phenylacetyl-CoA catabolon: a complex catabolic unit with broad biotechnological applications. *Mol Microbiol* **39**, 1434–1442.
- Luengo, J. M., Arias, S., Sandoval, A., Arias-Barrau, E., Arcos, M., Naharro, G. & Olivera, E. R. (2004). From aromatics to bioplastics: the phenylacetyl-CoA catabolon as a model of catabolic convergence. *Res Dev Bioph Biochem* **4**, 257–292.
- Martinez-Blanco, H., Reglero, A., Rodriguez-Aparicio, L. B. & Luengo, J. M. (1990). Purification and biochemical characterization of phenylacetyl-CoA ligase from *Pseudomonas putida*: a specific enzyme for the catabolism of phenylacetic acid. *J Biol Chem* **265**, 7084–7090.
- Miyamoto, S. & Tokuda, H. (2007). Diverse effects of phospholipids on lipoprotein sorting and ATP hydrolysis by the ABC transporter LolCDE complex. *Biochim Biophys Acta* **1768**, 1848–1854.
- Mooney, A., O'Leary, N. D. & Dobson, A. D. (2006a). Cloning and functional characterization of the *styE* gene, involved in styrene transport in *Pseudomonas putida* CA-3. *Appl Environ Microbiol* **72**, 1302–1309.
- Mooney, A., Ward, P. G. & O'Connor, K. (2006b). Microbial degradation of styrene: biochemistry, molecular genetics, and perspectives for biotechnological applications. *Appl Microbiol Biotechnol* **72**, 1–10.
- Narita, S.-i. & Tokuda, H. (2006). An ABC transporter mediating the membrane detachment of bacterial lipoproteins depending on their sorting signals. *FEBS Lett* **580**, 1164–1170.
- Nelson, K. E., Weinell, C., Paulsen, I. T., Dodson, R. J., Hilbert, H., Martins dos Santos, V. A., Fouts, D. E., Gill, S. R., Pop, M. & other authors (2002). Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. *Environ Microbiol* **4**, 799–808.
- Neuwald, A. F., Aravind, L., Spouge, J. L. & Koonin, E. V. (1999). A class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes. *Genome Res* **9**, 27–43.
- Nogales, J., Macchi, R., Franchi, F., Barzaghi, D., Fernandez, C., Garcia, J. L., Bertoni, G. & Diaz, E. (2007). Characterization of the last step of the aerobic phenylacetic acid degradation pathway. *Microbiology* **153**, 357–365.
- O'Connor, K., Buckley, C. M., Hartmans, S. & Dobson, A. D. W. (1995). Possible regulatory role for nonaromatic carbon sources in styrene degradation by *Pseudomonas putida* CA-3. *Appl Environ Microbiol* **61**, 544–548.
- O'Connor, K. E., Duetz, W., Wind, B. & Dobson, A. D. W. (1996). The effect of nutrient limitation on styrene metabolism in *Pseudomonas putida* CA-3. *Appl Environ Microbiol* **62**, 3594–3599.
- O'Connor, K. E., Witholt, B. & Duetz, W. (2001). *p*-Hydroxyphenylacetic acid metabolism in *Pseudomonas putida* F6. *J Bacteriol* **183**, 928–933.
- O'Leary, N. D., O'Connor, K. E., Duetz, W. & Dobson, A. D. W. (2001). Transcriptional regulation of styrene degradation in *Pseudomonas putida* CA-3. *Microbiology* **147**, 973–979.
- O'Leary, N. D., Duetz, W. A., Dobson, A. D. & O'Connor, K. E. (2002a). Induction and repression of the *sty* operon in *Pseudomonas putida* CA-3 during growth on phenylacetic acid under organic and inorganic nutrient-limiting continuous culture conditions. *FEMS Microbiol Lett* **208**, 263–268.
- O'Leary, N. D., O'Connor, K. E. & Dobson, A. D. W. (2002b). Biochemistry, genetics and physiology of microbial styrene degradation. *FEMS Microbiol Rev* **26**, 403–417.
- O'Leary, N. D., O'Connor, K. E., Ward, P., Goff, M. & Dobson, A. D. W. (2005). Genetic characterization of accumulation of polyhydroxyalkanoate from styrene in *Pseudomonas putida* CA-3. *Appl Environ Microbiol* **71**, 4380–4387.

- Olivera, E. R., Minambres, B., Garcia, B., Muntiz, C., Moreno, M. A., Ferrandez, A., Diaz, E., Garcia, J. L. & Luengo, J. M. (1998). Molecular characterization of the phenylacetic acid catabolic pathway in *Pseudomonas putida* U: the phenylacetyl-CoA catabolon. *Proc Natl Acad Sci U S A* **95**, 6419–6424.
- O'Toole, G. A. & Kolter, R. (1998). Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. *Mol Microbiol* **28**, 449–461.
- Park, S. J. & Lee, S. Y. (2005). Systems biological approach for the production of various polyhydroxyalkanoates by metabolically engineered *Escherichia coli*. *Macromol Symp* **224**, 1–10.
- Park, J. H., Lee, S. Y., Kim, T. Y. & Kim, H. U. (2008). Application of systems biology for bioprocess development. *Trends Biotechnol* **26**, 404–412.
- Patel, C. N., Wortham, B. W., Lines, J. L., Fetherston, J. D., Perry, R. D. & Oliveira, M. A. (2006). Polyamines are essential for the formation of plague biofilm. *J Bacteriol* **188**, 2355–2363.
- Patrauchan, M. A., Sarkisova, S. A. & Franklin, M. J. (2007). Strain-specific proteome responses of *Pseudomonas aeruginosa* to biofilm-associated growth and to calcium. *Microbiology* **153**, 3838–3851.
- Pötter, M. & Steinbüchel, A. (2005). Poly(3-hydroxybutyrate) granule-associated proteins: impacts on poly(3-hydroxybutyrate) synthesis and degradation. *Biomacromolecules* **6**, 552–560.
- Pötter, M., Muller, H., Reinecke, F., Wiecek, R., Fricke, F., Bowien, B., Friedrich, B. & Steinbüchel, A. (2004). The complex structure of polyhydroxybutyrate (PHB) granules: four orthologous and paralogous phasins occur in *Ralstonia eutropha*. *Microbiology* **150**, 2301–2311.
- Raberg, M., Reinecke, F., Reichelt, R., Malkus, U., König, S., Pötter, M., Fricke, W. F., Pohlmann, A., Voigt, B. & other authors (2008). *Ralstonia eutropha* H16 flagellation changes according to nutrient supply and state of poly(3-hydroxybutyrate) accumulation. *Appl Environ Microbiol* **74**, 4477–4490.
- Raghava, G. P. S. (2006). MANGO: prediction of Genome Ontology (GO) class of a protein from its amino acid and dipeptide composition using nearest neighbor approach. *CASP7* **93**.
- Ramos, J. L., Gallegos, M.-T., Marqués, S., Ramos-González, M.-I., Espinosa-Urgel, M. & Segura, A. (2001). Responses of Gram-negative bacteria to certain environmental stressors. *Curr Opin Microbiol* **4**, 166–171.
- Ramos, J. L., Duque, E., Gallegos, M.-T., Godoy, P., Ramos-González, M. I., Rojas, A., Terán, W. & Segura, A. (2002). Mechanisms of solvent tolerance in Gram-negative bacteria. *Annu Rev Microbiol* **56**, 743–768.
- Ramos-Gonzalez, M.-I., Ruiz-Cabello, F., Brettrich, I., Garrido, F. & Ramos, J. L. (1992). Tracking genetically engineered bacteria: monoclonal antibodies against surface determinants of the soil bacterium *Pseudomonas putida* 2440. *J Bacteriol* **174**, 2978–2985.
- Rehm, B. H. (2007). Biogenesis of microbial polyhydroxyalkanoate granules: a platform technology for the production of tailor-made bioparticles. *Curr Issues Mol Biol* **9**, 41–62.
- Sandoval, A., Arias-Barrau, E., Arcos, M., Naharro, G., Olivera, E. R. & Luengo, J. M. (2007). Genetic and ultrastructural analysis of different mutants of *Pseudomonas putida* affected in the poly-3-hydroxy-n-alkanoate gene cluster. *Environ Microbiol* **9**, 737–751.
- Santos, P. M., Benndorf, D. & Sa-Correia, I. (2004). Insights into *Pseudomonas putida* KT2440 response to phenol-induced stress by quantitative proteomics. *Proteomics* **4**, 2640–2652.
- Santos, P. M., Roma, V., Benndorf, D., von Bergen, M., Harms, H. & Sa-Correia, I. (2007). Mechanistic insights into the global response to phenol in the phenol-biodegrading strain *Pseudomonas* sp. M1 revealed by quantitative proteomics. *OMICS* **11**, 233–251.
- Scheiner, D. (1976). Determination of ammonia and Kjeldahl nitrogen by indophenol method. *Water Res* **10**, 31–36.
- Schlegel, H. G., Kaltwasser, H. & Gottschalk, G. (1961). A submersion method for culture of hydrogen-oxidizing bacteria: growth physiological studies. *Arch Mikrobiol* **38**, 209–222 in German.
- Shevchenko, A., Wilm, M., Vorm, O. & Mann, M. (1996). Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal Chem* **68**, 850–858.
- Smith, J. L. (2004). The physiological role of ferritin-like compounds in bacteria. *Crit Rev Microbiol* **30**, 173–185.
- Sobolevsky, T. G., Revelsky, A. I., Revelsky, I. A., Miller, B. & Oriedo, V. (2004). Simultaneous determination of fatty, dicarboxylic and amino acids based on derivatization with isobutyl chloroformate followed by gas chromatography-positive ion chemical ionization mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci* **800**, 101–107.
- Squires, C. & Squires, C. L. (1992). The Clp proteins: proteolysis regulators or molecular chaperones? *J Bacteriol* **174**, 1081–1085.
- Taylor, M., Tuffin, M., Burton, S., Eley, K. & Cowan, D. (2008). Microbial responses to solvent and alcohol stress. *Biotechnol J* **3**, 1388–1397.
- Tessmer, N., König, S., Malkus, U., Reichelt, R., Pötter, M. & Steinbüchel, A. (2007). Heat-shock protein HspA mimics the function of phasins *sensu stricto* in recombinant strains of *Escherichia coli* accumulating polythioesters or polyhydroxyalkanoates. *Microbiology* **153**, 366–374.
- Velasco, A., Alonso, S., Garcia, J. L., Perera, J. & Diaz, E. (1998). Genetic and functional analysis of the styrene catabolic cluster of *Pseudomonas* sp. strain Y2. *J Bacteriol* **180**, 1063–1071.
- VerBerkmoes, N. C., Shah, M. B., Lankford, P. K., Pelletier, D. A., Strader, M. B., Tabb, D. L., McDonald, W. H., Barton, J. W., Hurst, G. B. & other authors (2006). Determination and comparison of the baseline proteomes of the versatile microbe *Rhodospseudomonas palustris* under its major metabolic states. *J Proteome Res* **5**, 287–298.
- Volker, U., Engelmann, S., Maul, B., Roethdorf, S., Voelker, A. & Schmid, R. (1994). Analysis of the induction of stress proteins of *Bacillus subtilis*. *Microbiology* **140**, 741–752.
- Volkers, R. J. M., de Jong, A. L., Hulst, A. G., van Baar, B. L. M., de Bont, J. A. M. & Wery, J. (2006). Chemostat-based proteomic analysis of toluene-affected *Pseudomonas putida* S12. *Environ Microbiol* **8**, 1674–1679.
- von Wallbrunn, A., Richnow, H. H., Neumann, G., Meinhardt, F. & Heipieper, H. J. (2003). Mechanism of *cis-trans* isomerization of unsaturated fatty acids in *Pseudomonas putida*. *J Bacteriol* **185**, 1730–1733.
- Wältermann, M. & Steinbüchel, A. (2005). Neutral lipid bodies in prokaryotes: recent insights into structure, formation, and relationship to eukaryotic lipid depots. *J Bacteriol* **187**, 3607–3619.
- Ward, P. G. & O'Connor, K. E. (2005). Induction and quantification of phenylacetyl-CoA ligase enzyme activities in *Pseudomonas putida* CA-3 grown on aromatic carboxylic acids. *FEMS Microbiol Lett* **251**, 227–232.
- Ward, P. G., de Roo, G. & O'Connor, K. E. (2005). Accumulation of polyhydroxyalkanoate from styrene and phenylacetic acid by *Pseudomonas putida* CA-3. *Appl Environ Microbiol* **71**, 2046–2052.
- Ward, P. G., Goff, M., Donner, M., Kaminsky, W. & O'Connor, K. E. (2006). A two step chemo-biotechnological conversion of polystyrene to a biodegradable thermoplastic. *Environ Sci Technol* **40**, 2433–2437.
- Washburn, M. P., Ulaszek, R. R. & Yates, J. R. I. (2003). Reproducibility of quantitative proteomic analyses of complex

biological mixtures by multi-dimensional protein identification technology. *Anal Chem* **75**, 5054–5061.

Welinder, K. G. (1991). Bacterial catalase-peroxidases are gene duplicated members of the plant peroxidase superfamily. *Biochim Biophys Acta* **1080**, 215–220.

Whang, L.-M., Liu, P.-W. G., Ma, C.-C. & Cheng, S.-S. (2008). Application of biosurfactants, rhamnolipid, and surfactin, for enhanced biodegradation of diesel-contaminated water and soil. *J Hazard Mater* **151**, 155–163.

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