RESEARCH ARTICLE

Adaptation to β-myrcene catabolism in *Pseudomonas* sp. M1: An expression proteomics analysis

*Pedro M. Santos** *and Isabel Sá-Correia*

IBB-Institute for Biotechnology and Bioengineering, Centre for Biological and Chemical Engineering, Instituto Superior Técnico, Lisboa, Portugal

Received: May 18, 2009
Revised: July 15, 2009
Accepted: August 25, 2009

β-Myrcene, a monoterpene widely used as a fragrance and flavoring additive, also possesses analgesic, anti-mutagenic, and tyrosinase inhibitory properties. In order to get insights into the molecular mechanisms underlying the ability of *Pseudomonas sp.* M1 to catabolize β-myrcene, an expression proteomics approach was used in this study. Results indicate that the catabolic enzyme machinery for β-myrcene utilization (MyrB, MyrC, and MyrD and other uncharacterized proteins) is strongly induced when β-myrcene is present in the growth medium. Since an M1 mutant, lacking a functional 2-methylisocitrate dehydratase, is not able to grow in mineral medium with β-myrcene or propionic acid as the sole C-source, and also based on the expression proteomic analysis carried out in this study, it is suggested that the β-myrcene catabolic intermediate propionyl-CoA is channeled into the central metabolism *via* the 2-methylcitrate cycle. Results also suggest that the major alteration occurring in the central carbon metabolism of cells growing in β-myrcene-containing media is related with the redistribution of the metabolic fluxes leading to increased oxaloacetate production. Other up-regulated proteins are believed to prevent protein misfolding and aggregation or to play important structural roles, contributing to the adaptive alteration of cell wall and membrane organization and integrity, which are essential features to allow the bacterium to cope with the highly lipophilic β-myrcene as C-source.

Keywords:
β-Myrcene / β-Myrcene catabolism / Essential oils / Expression proteomics / Microbiology / *Pseudomonas*

1 Introduction

Plants produce a very large diversity of metabolites resulting from the differential modification of common backbone structures, catalyzed by different enzymes [1]. A large percentage of plant metabolites are terpenoids, also known as isoprenoids because they are synthesized through the condensation of C_5 isoprene units. Monoterpenes and sesquiterpenes represent the C_{10} and C_{15} terpene classes, respectively. Many of these compounds are volatiles, which have flavor/aroma properties, are toxic, and play important roles in plant defence, plant-to-plant communication, and pollinator attraction [2]. A number of terpenoids have recognized therapeutic properties: anti-cancer, analgesic, anti-malarial, anti-ulcer, hepaticidal, anti-microbial, anti-mutagenic, and diuretic activities [3]. Because of their diversity and distinct biological roles, the commercial and ecological importance of terpenoids makes their metabolic engineering envisaging the improvement of their value an attractive topic of research [4].

The acyclic monoterpene β-myrcene (7-methyl-3-methylene-1,6-octadiene) is a highly hydrophobic organic compound (octanol/water partition coefficient, log Kow: 4.17 [5]) that hardly dissolves in the aqueous phase (water

Correspondence: Dr. Pedro M. Santos, IBB-Institute for Biotechnology and Bioengineering, Centre for Biological and Chemical Engineering, Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisboa, Portugal
E-mail: pedrosantos@ist.utl.pt
Fax: +351-218419199

*Current address: CBMA-Centre of Molecular and Environmental Biology, Department of Biology, University of Minho, Campus de Gualtar, 4715-057 Braga, Portugal.

solubility at 25°C approx. 6 mg/L [5]). Myrcene is found in essential oils from a variety of plants, including lemongrass, verbena, hop, and bay [6]. β-Myrcene or essential oils containing this terpenoid are widely used as fragrance, in cosmetics and household products, and as flavoring additive in food and alcoholic beverages [6]. In addition, β-myrcene possesses analgesic [7], anti-mutagenic [6], and tyrosinase inhibitory [8] properties. The environmental strain *Pseudomonas* sp. M1, isolated from the Rhine River sediments [9] can use β-myrcene as sole carbon and energy source [9] and is also able to utilize several toxic and/or recalcitrant compounds as sole carbon and energy sources, including phenol and benzene [10–12]. Beside this, M1 strain, only *Pseudomonas putida* S4-2 was reported to be able to oxidize β-myrcene, more than two decades ago [13]. However, no further work was, apparently, carried out to characterize β-myrcene oxidation ability of S4-2 strain. Therefore, *Pseudomonas* sp. M1 can be considered the only well-described bacterium able to utilize β-myrcene as the sole carbon source [9]. A phylogenetic analysis indicated that *P. citronellolis* is the closest species related to *Pseudomonas* sp. M1 [11]. The genes *myrA*, *myrB*, *myrC*, and *myrD*, coding for an aldehyde dehydrogenase, an alcohol dehydrogenase, CoA ligase, and enoyl-CoA hydratase, respectively, were implicated before in the catabolism of β-myrcene in *Pseudomonas* sp. M1 [9]. The knockout of the *myrB* gene resulted in the loss of M1 ability to growusing β-myrcene as sole carbon source and in the accumulation of (*E*)-2-methyl-6-methylene-2,7-octadiene-1-ol [9]. Based on these results, the catabolic pathway of β-myrcene in *Pseudomonas* sp. M1 was proposed: β-myrcene is believed to be firstly hydroxylated and, following intermediate activation of 2-methyl-6-methylene-2,7-octadienoic acid by the CoA ligase MyrC, the β-myrcene catabolic pathway proceeds *via* a β-oxidation-like pathway [9].

In this study, we combined molecular biology approaches and expression proteomics to obtain a global insight into the adaptive response to β-myrcene catabolism in *Pseudomonas* sp. M1. This study focuses on the required catabolic enzymes on the regulation of β-myrcene catabolism and on the global cell response to this hydrophobic compound.

2 Materials and methods

2.1 Bacterial strains, media, and growth conditions

Pseudomonas sp. M1 [9] and the transposon-mutant *Pseudomonas* sp. M1-2, obtained during this study, were cultivated in 50-mL mineral medium [14] in 250 mL Erlenmeyer-sealed flasks, with orbital shaking (250 rpm), at 30°C, using as carbon sources: (i) 40 mM pyruvate, (ii) 40 μL β-myrcene (CAS number: 123-35-3; density: 0.796 g/mL; log Kow: 4.17; water solubility at 25°C approx. 6 mg/L [5]), which results in a β-myrcene saturating concentration in the aqueous phase, or (iii) 40 mM pyruvate plus 40 μL β-myrcene. For β-myrcene-induction studies, inocula were prepared by diluting pyruvate-grown cell culture samples (at 600 nm (OD_{600}) in the range 0.5–0.6) with fresh mineral medium containing pyruvate to obtain an initial OD_{600} of the main culture of 0.05 ± 0.005. Cultures were incubated until an OD_{600} of 0.2 (in the exponential phase) was reached and β-myrcene (40 μL) was added to the cultures and incubation proceeded for an additional period of about 12 h. Growth medium pH was determined, in triplicate samples, using a glass electrode and pH meter.

Luria Bertani medium was used to grow *Pseudomonas* and *Escherichia coli* strains to obtain cells for molecular biology experiments. When necessary, culture media were supplemented with ampicillin (Ap, 100 μg/mL) or kanamycin (Km, 50 μg/mL), to maintain the selective pressure over the transformants.

2.2 Transposon mutagenesis and identification of chromosomal insertion regions

The mini-transposon pUTKm [15] was mobilized from *E. coli* CC118λpir to *Pseudomonas* sp. M1 by triparental mating, as described earlier [15]. Replica plating was used to screen those transconjugants that had lost the ability to grow on β-myrcene as sole carbon source, which were isolated after 48 h incubation in β-myrcene-mineral medium. Chromosomal DNA extracted from the selected transposon insertion mutants was used for the construction of a genomic library using pWEB::TNC cosmid cloning kit (Epicentre, Wisconsin, WI, USA). DNA extraction, restriction, and ligation were performed using standard procedures [16]. The chromosomal insertion regions of the mini-transposon in the selected mutants were determined by applying arbitrary primed PCR, followed by sequencing of the resulting fragments, as described earlier [12].

2.3 Preparation of crude cell extracts and protein quantification

Cell samples harvested from the different cultures were used for expression proteomic analysis based on 2-DE separations. In order to reduce the effects of biological variation in the different experiments carried out, three cell samples obtained from three independent and identical growth experiments were pooled, centrifuged ($8000 \times g$ for 10 min at 4°C), resuspended in the sonication buffer (10 mM Tris base), and disrupted by sonication (five treatments of 15 s with 15 s intervals on ice between treatments). Two pooled samples for each condition were used. Cell debris was removed by centrifugation ($10\,000 \times g$ for 10 min at 4°C) and lysates were stored in aliquots at −70°C. Crude cell extract proteins were quantified by the bicinchoninic acid assay according to the instructions provided in the Pierce quantification kit, using BSA as the standard.

2.4 2-D polyacrylamide gel electrophoresis

The proteomes of *Pseudomonas* sp. M1 were separated by 2-D polyacrylamide gel electrophoresis (2-DE), essentially as described previously [11, 17] but using a cup-loading rather than a rehydration loading protocol. The protein extracts from cell samples grown in pyruvate medium either or not supplemented with β-myrcene and protein extracts from cells grown in β-myrcene as sole carbon source were used. In each separation, 50 μg of protein extract were used and a reference sample was run in parallel with the samples under study. This reference sample was prepared by pooling together 1 mg of protein extract obtained from each different biological sample under study. Since the second dimension of the 2-DE separation was carried out using an Ettan Daltsix system (GE Healthcare), and to facilitate inter-gel protein matching, each run contained a reference sample (in duplicate) and two independent samples (in duplicate) for the same condition. In total, eight gels for each condition were obtained and analyzed. Protein spots were detected by Flamingo fluorescent gel staining according to the instructions provided by the manufacturer (Bio-Rad).

2.5 Quantification of protein expression levels

The gels were scanned (Typhoon Trio; GE Healthcare) and the corresponding gel images were analyzed using Progenesis SameSpots v3.1 (Nonlinear Dynamics) software. Protein spots were identified by using the automatic spot detection algorithm. Individual spot volumes were normalized against total spot volumes for a given gel. Averages for each growth condition were then compared by their normalized volume using one-way ANOVA between-group test. Only statistically significant spots ($p < 0.05$) were selected for analysis. Differential expression between different experimental conditions was quantified and a threshold of at least a 1.5-fold increase or 0.6-fold decrease between averaged gels was considered. Spots that showed evidence of saturation were not included for further analysis.

2.6 Identification of proteins

Several protein spots were identified in the course of a previous work [11]. Nevertheless, the identification of several important protein spots was confirmed during this study. The identification of new spots of possible interest was also performed by peptide mass fingerprint in the proteomics unit at the CNIC Foundation (Centro Nacional de Investigaciones Cardiovasculares Carlos III, Madrid, Spain), as a paid service. Protein spots were excised manually from polyacrylamide gels and then digested automatically using a Proteineer DP protein digestion station (Bruker-Daltonics, Bremen, Germany). The digestion protocol used was that of Shevchenko *et al.* [18] with minor variations: gel plugs were submitted to reduction with 10 mM DTT (GE Healthcare) in 50 mM ammonium bicarbonate (99.5% purity; Sigma Chemical, St. Louis, MO, USA) and alkylation with 55 mM iodoacetamide (Sigma Chemical) in 50 mM ammonium bicarbonate. The gel pieces were then rinsed with 50 mM ammonium bicarbonate and ACN (gradient grade; Merck, Darmstadt, Germany) and dried under a stream of nitrogen. Modified porcine trypsin (sequencing grade; Promega, Madison, WI, USA) at a final concentration of 13 ng/μL in 50 mM ammonium bicarbonate was added to the dry gel pieces and the digestion proceeded at 37°C for 6 h. Finally, 0.5% TFA (99.5% purity; Sigma Chemical) was added for peptide extraction. An aliquot of the above digestion solution was mixed with an aliquot of CHCA (Bruker-Daltonics) in 33% aqueous ACN and 0.1% TFA. This mixture was deposited onto a 600 μm AnchorChip MALDI probe (Bruker-Daltonics) and allowed to dry at room temperature. MALDI-MS(/MS) data were obtained using an Ultraflex TOF mass spectrometer (Bruker-Daltonics) equipped with a LIFT-MS/MS device [19]. Spectra were acquired in the positive-ion mode at 50 Hz laser frequency, and 100–1500 individual spectra were averaged. For fragment ion analysis in the TOF/TOF mode, precursors were accelerated to 8 kV and selected in a timed ion gate. Fragment ions generated by laser-induced decomposition of the precursor were further accelerated by 19 kV in the LIFT cell and their masses were analyzed after passing the ion reflector. Measurements were in part performed using post-LIFT metastable suppression, which allowed removal of precursor and metastable ion signals produced after extraction out of the second ion source. Detailed analysis of peptide mass mapping data was performed using flexAnalysis software (Bruker-Daltonics). Internal calibration of MALDI-TOF mass spectra was performed using two trypsin autolysis ions with $m/z = 842.510$ and $m/z = 2211.105$; for MALDI-MS/MS, calibrations were performed with fragment ion spectra obtained for the proton adducts of a peptide mixture covering the m/z 800–3200 region. MALDI-MS and MS/MS data were combined through MS BioTools program (Bruker-Daltonics) to search the NCBInr database using MASCOT software (Matrix Science, London, UK) [20].

3 Results and discussion

3.1 Metabolization of β-myrcene by *Pseudomonas* sp. M1

Cells of *Pseudomonas* sp. M1, pre-adapted to growth using β-myrcene as the only carbon source, are able to grow in β-myrcene-mineral medium exhibiting a maximum specific growth rate (μ_{max}) of about 0.73/h (Fig. 1A). The transition from the exponential phase to the stationary phase of growth was found to occur at a culture OD_{600} between 1.3 and 1.6, coinciding with a strong decrease of growth medium pH (Fig. 1A). This decrease of pH continued

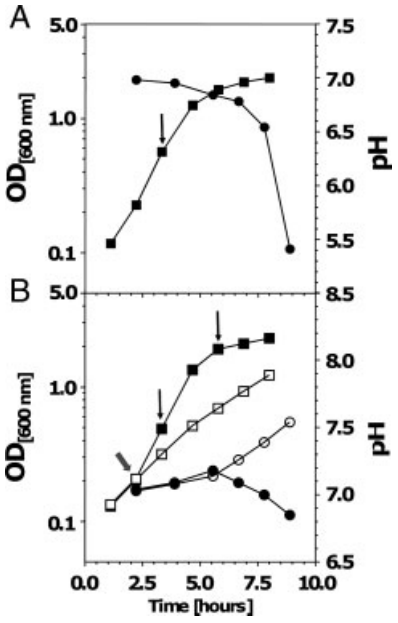

Figure 1. Representative growth curves (squares) of *Pseudomonas* sp. M1 and variation of growth medium pH (circles) when growth media with different carbon sources was used. Cells were grown in mineral medium with β-myrcene as sole carbon source (A) or with pyruvate supplemented (closed symbols) or not supplemented (open symbols) with 40 μL of β-myrcene, at the time point indicated by the grey arrow (B). Time of sampling for proteome comparison is indicated by the black arrow. For each condition, three independent growth experiments were carried out in duplicate.

during stationary phase reaching a pH value of 4.3 ± 0.2 after 12 h of cultivation during the stationary phase (data not shown), possibly due to extracellular accumulation of organic acids [21, 22] resulting from β-myrcene catabolism.

In order to compare the effects of the presence of β-myrcene in the medium of culture of *Pseudomonas* sp. M1 or of derived mutants (generated by random transposon mutagenesis) that have lost the ability to grow in β-myrcene as sole carbon source, a pyruvate-based medium was selected to conduct these studies. In fact, in the previous works pyruvate was shown to be the less repressing carbon source when a less easily metabolizable carbon source is also present in the medium, as alternative C-source for *Pseudomonas* sp. M1 [10, 11].

When *Pseudomonas* sp. M1 was cultivated in mineral media with pyruvate as the sole C-source, the μ_{max} was around 0.45/h and growth medium pH increased up to approximately 7.6. However, when an identical pyruvate-grown cell culture was supplemented with β-myrcene, at an OD_{600} of 0.2, the μ_{max} increased from μ_{max}0.45/h to μ_{max}0.85/h and a higher final biomass concentration was obtained, suggesting the co-metabolization of pyruvate and β-myrcene when both carbon sources are present in the growth medium (Fig. 1B). After 3 h of incubation following β-myrcene addition, the growth medium pH was about 7.2 and started to decrease to around 6.8. This value remained constant for, at least, 12 h of cultivation (data not shown). This effect of β-myrcene metabolism on medium pH is likely to be partially responsible for the higher final biomass obtained in the culture medium with both pyruvate and β-myrcene as C-source.

Following random transposon mutagenesis, envisaging the identification of genes involved in β-myrcene catabolism in *Pseudomonas* sp. M1, two different mutants unable to grow in β-myrcene were selected. One of the mutants had the *Km*-cassette inserted within a gene homologous to a transcriptional regulator of the LuxR family. In the other mutant (denominated M1-2), the *Km*-cassette was inserted into a gene homologous to the *acnD* gene of *P. aeruginosa* PA7 (accession number: ABR85027). This gene codes for a 2-methylisocitrate dehydratase (AcnD) that catalyzes the conversion of 2-methylaconitate to 2-methylisocitrate (Fig. 2A). In *P. aeruginosa* PA7 and in other *Pseudomonas* strains, the *acnD* gene is localized within a genetic locus, as shown in Fig. 2B, involved in the 2-methylcitrate cycle which is the predominant pathway for propionic acid catabolism in Gram-negative bacteria [23]. The first intermediate of propionic acid catabolic pathway [23] is propionyl-CoA, which is also a putative metabolite of β-myrcene catabolism [9] (Fig. 2A). Consistent with the hypothesized pathway for propionic acid and β-myrcene catabolism, the mutant M1-2 (Km::*acnD*) was found to be unable to grow in mineral medium with propionic acid as sole C-source (data not shown). However, the mutant *Pseudomonas* sp. M1-2 exhib-

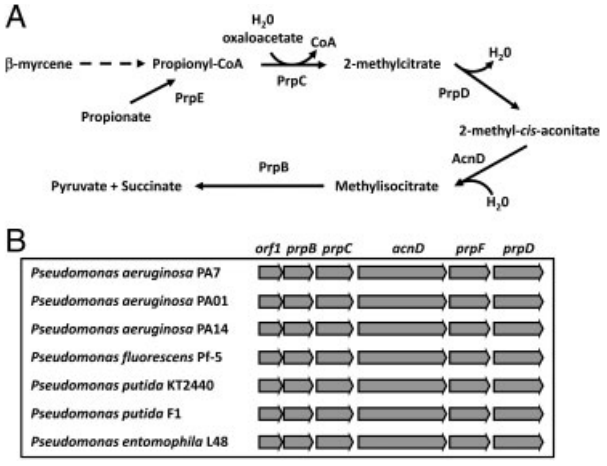

Figure 2. The putative propionyl-CoA pathway of *Pseudomonas* sp. M1. (A) Schematic representation of the hypothesized propionyl-CoA pathway, based on the results reported in this study and on Metacyc database (http://metacyc.org/). (B) Gene organization in the genomic locus coding for the propionyl-CoA pathway in several *Pseudomonas* strains whose genome sequence is available, based on the Pseudomonas Genome database (http://www.pseudomonas.com). *prpB*, 2-methylcitrate lyase; *prpC*, 2-methylcitrate synthase; *acnD*, 2-methylisocitrate dehydratase/aconitase A; *prpF*, probable AcnD-accessory protein and *prpD*, 2-methylcitrate dehydratase.

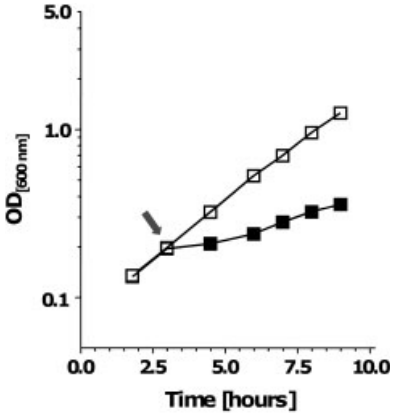

Figure 3. Representative growth curves of *Pseudomonas* sp. M1-2 (M1 mutant with the *acnD* gene interrupted with a kanamycin resistance cassette) in pyruvate-mineral medium supplemented (closed symbols) or not (open symbols) with β-myrcene, at the time point indicated by the grey arrow.

ited a maximum specific growth rate (μ_{max}0.45/h) identical to the wild-type strain when grown in mineral media with pyruvate as the sole C-source (Fig. 3) while this μ_{max} was strongly reduced by 80% when this mutant was cultivated in this same medium supplemented with β-myrcene. This observation suggests that a toxic intermediate metabolite from β-myrcene catabolism might accumulate in the *acnD* knockout mutant. Mutants of *P. putida* KT2440 or *Ralstonia eutropha* H16 with genes homologous to *acnD* deleted are known to accumulate 2-methylcitrate when grown in propionate or levulinate [23]. This metabolite exerts a strong inhibitory effect both in prokaryotes and in eukaryotes and has been proposed as a potential inhibitor for fast growing cells in cancer research [23].

3.2 β-Myrcene-dependent alteration of the proteome of *Pseudomonas* sp. M1

An expression proteomics analysis was carried out to quantify the expression levels of the enzymes involved in β-myrcene catabolism in *Pseudomonas* sp. M1 and to get a snapshot of β-myrcene-dependent alteration of the proteome of M1, expecting to unveil the molecular mechanisms involved in M1 adaptation to growth on β-myrcene. The proteomes of cell samples obtained during *Pseudomonas* sp. M1 cultivation in mineral medium with (i) pyruvate, (ii) β-myrcene or (iii) pyruvate and β-myrcene, as C-sources (Fig. 1), were compared. In order to facilitate inter-condition proteome comparison, a reference sample was generated by mixing, into a single vial, identical amounts of the total protein extracts prepared from cell samples obtained from each growth condition. This reference sample contained all the proteins expressed in any of the growth conditions tested and was used to prepare a protein reference map for *Pseudomonas* sp. M1. Although a reference map for *Pseudomonas* sp. M1 proteome [11] has been generated before, in this study the proteins were focused using a cup-loading rather than a rehydration solution protocol and the in-gel relative mobility of some of the previously identified proteins was, apparently, modified. A significant percentage of the protein spots of interest could not be identified, probably due to the absence of significantly homologous proteins in the databases. The *Pseudomonas* sp. M1 reference map prepared in this study and the corresponding list of identified proteins are given in Supporting Information. Among all the 2-DE gels used in this expression proteomic analysis, an average of 1315 protein spots were detected, quantified and compared. The most important responses that emerged from this study, based on the expression modifications induced by β-myrcene, are detailed below.

3.2.1 β-Myrcene induction of catabolic enzymes

Eleven highly abundant protein spots were detected in 2-DE gels using extracts prepared from cells grown in β-myrcene-supplemented media, suggesting their involvement in β-myrcene catabolism. These proteins were associated to numbers 1 to 10 and to number 25 in the Supporting Information Table A. The relative abundance of the referred proteins, in M1 cells grown in β-myrcene or in pyruvate mineral media, either or not supplemented with β-myrcene, was quantified following separation in 2-DE gels (Table 1). This group of proteins includes MyrB, MyrC and MyrD, implicated in β-myrcene catabolism [9], and AcnD which, according to the results obtained with the knockout mutant in *acnD* (Section 3.1), is proposed to be also required for β-myrcene catabolism. Results indicate that the enzymes involved in β-myrcene catabolic pathway are only induced when β-myrcene is present in growth medium. The relative abundance of other protein spots whose identification by MS has failed but that was found to be strongly induced by β-myrcene, which is also summarized in Table 1. Representative images of the strong variation registered in the relative abundance of β-myrcene catabolic enzymes in response to the presence of β-myrcene in the growth medium are shown in Fig. 4. The β-myrcene-dependent induction fold ranged from 4- to 77-fold, when cells were grown in pyruvate-medium supplemented with β-myrcene or in medium with β-myrcene as sole carbon source. Under the experimental conditions used in this study, a higher induction fold was in general registered when cells were cultivated for 3 h in pyruvate medium supplemented with β-myrcene (PM3/Pyr) compared with cells cultivated for only 1 h following β-myrcene supplementation (PM1/Pyr) (Table 1). Results also indicate that the expression of proteins encoded by genes putatively organized in polycistronic structures is induced at similar levels. This is the case of MyrB and MyrC proteins, encoded by *Pseudomonas* sp. M1 *myrABC* operon

Table 1. Expression fold change of β-myrcene catabolic enzymes when cells of *Pseudomonas* sp. M1 were grown in β-myrcene supplemented media (Myr, PM1 or PM3) compared with cells grown in mineral medium with pyruvate as sole carbon source (Pyr)

Spot	Protein	Fold change[a)]			ANOVA (*p*)
		Myr/Pyr[a)]	PM1/Pyr[a)]	PM3/Pyr[a)]	
1	Alcohol dehydrogenase, MyrB	19.4	9.6	9.5	1.22E−05
2	Acyl-CoA ligase, MyrC	17.9	8.6	10.6	2.30E−12
3	Enoyl-CoA hydratase, MyrD	17.8	13.5	21.4	3.33E−16
4	Acyl-CoA dehydrogenase	44.8	26.0	50.3	3.95E−13
5	Short chain enoyl-CoA hydratase	18.7	11.1	13.9	2.57E−13
6	Dioxygenase	50.0	28.8	38.6	4.12E−12
7	Dioxygenase	8.9	7.3	10.8	1.03E−05
8	Hypothetical protein precursor	17.5	9.1	15.6	6.33E−12
9	2-Methylisocitrate dehydratase	9.8	7.7	7.8	2.52E−10
10	2-Methylisocitrate lyase	9.6	7.2	10.4	2.78E−15
25	2-Methylcitrate synthase	10.7	7.9	10.3	0.006669
103[b)]	–	12.3	6.7	12.8	0.000317
105[b)]	–	77.2	32.4	53.1	3.15E−05
109[b)]	–	15.6	10.5	15.4	1.12E−05
113[b)]	–	8.7	5.5	12.4	0.000517
119[b)]	–	8.0	4.2	6.3	3.12E−05
121[b)]	–	9.7	5.9	10.7	8.07E−10

Myr, β-myrcene grown cells; Pyr, pyruvate grown cells; PM1, cells grown in pyruvate medium and β-myrcene for 1 h; PM3, cells grown in pyruvate medium and β-myrcene for 3 h.

a) Fold change was calculated as: [(spot-normalized volume in samples grown inβ-myrcene-containing media)/(spot-normalized volume in reference sample)]/[(spot-normalized volume in pyruvate-grown cells)/(spot-normalized volume in reference sample)]. Reference sample (also known as internal standard) was obtained by mixing equal amounts of proteins from all tested conditions. Each spot normalized volume corresponds to values obtained from four 2-DE gels analysis.

b) Protein spots that could not be identified.

[9] and of PrpB, PrpC and AcnD proteins known to be are organized, in other biological systems, in an operonic structure [23]. The *in silico* analysis of the *prp* locus was carried out in this study, using the available genome sequences of *Pseudomonas* species/strains at the *Pseudomonas* Genome Database (www.pseudomonas.com) (Fig. 2B). This analysis indicates that the gene organization at the *prp* locus is apparently conserved in *Pseudomonas*.

Based on the β-myrcene catabolic pathway proposed earlier [9] and on the inferred functions of the proteins identified in this study, it is likely that the 3-hydroxyacyl-CoA dehydrogenase enzyme (spot 5) is involved in the conversion of the product of MyrD catabolic activity into the 2-methyl-3-keto-6-methylene-7-octenoyl-CoA intermediate. However, this hypothesis has to be proved. The specific catabolic reactions catalyzed by β-myrcene-induced dioxygenase (spots 6 and 7) and by acyl-CoA dehydrogenase (spot 4) are still unclear. In *P. aeruginosa* strains whose genome sequence is known, the gene-encoding proteins corresponding to the referred spots are organized in the same operonic structure, as predicted by the Pathologic tool available in *Pseudomonas* Genome Database (data not shown). These enzymes are presumably involved in fatty acid degradation, which is consistent with the proposed β-myrcene catabolism *via* a β-oxidation-like pathway.

3.2.2 β-Myrcene-induced alterations related to central energetic metabolism

Twenty-four different proteins, related to central energetic metabolism, were mapped in the proteome of *Pseudomonas* sp. M1 (Supporting Information Table A). An altered expression in response toβ-myrcene was registered for five of these proteins (above 1.5-fold or below 0.6-fold compared with expression in pyruvate-grown cells) (Table 2). With one exception, these proteins are involved in the metabolism of oxaloacetate, a hypothesized metabolite essential for channelingβ-myrcene intermediates into central metabolism (Fig. 5). The abundance of pyruvate carboxylase, subunit B (PycB) and phosphoenolpyruvate carboxykinase (PckA), involved in oxaloacetate biosynthesis [24], was found to increase, whereas the abundance of citrate synthase (GltA), involved in oxaloacetate consumption, was found to decrease in cells grown inβ-myrcene-containing media [24]. In cells grown inβ-myrcene-containing media, the production of acetyl-CoA, *via* the pyruvate dehydrogenase complex, is likely to be reduced, as suggested by the decreased abundance of the pyruvate dehydrogenase complex subunit AceE (Table 2). These results are consistent with the redistribution of the metabolic fluxes toward an increased production of oxaloacetate inβ-myrcene-grown cells.

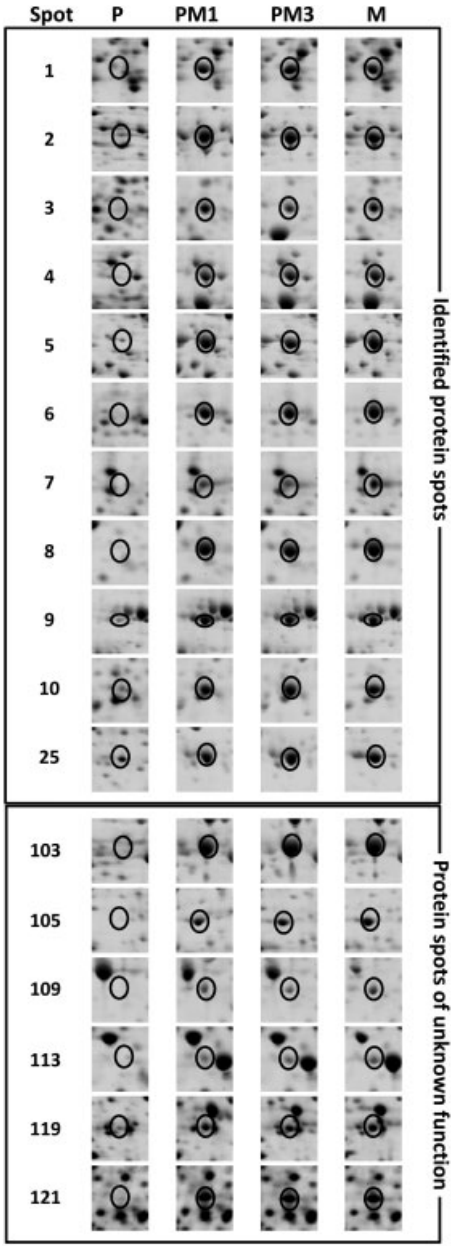

Figure 4. Representative images of protein spots corresponding to β-myrcene catabolic enzymes in: P, pyruvate-grown cells; PM1, cells grown for 1 h in pyruvate medium supplemented with β-myrcene; PM3, cells grown for 3 h in pyruvate medium supplemented with β-myrcene; M, β-myrcene-grown cells. The legend of the numbered spots (1–10, 25) is provided in Supporting Information Table A.

3.2.3 β-Myrcene-induced alteration of the content of proteins involved in the transport of small molecules

One of the most striking features of β-myrcene catabolism in *Pseudomonas* sp. M1 concerns the mechanisms employed by this bacteria for sensing β-myrcene and on how it becomes available for utilization as C-source. β-Myrcene is a highly hydrophobic organic compound (Log $K_{OW} = 4.17$ [5]) that hardly dissolves in the aqueous phase. It is likely that β-myrcene primarily accumulates at the level of the biological membrane, as demonstrated for other terpenes [25]. In general, terpene interaction with the hydrophobic part of the membrane affects membrane anisotropy and dipolar organization [25] and may also involve membrane receptor-mediated effects [25]. The relative abundance of six out of the nine different proteins involved in the transport of small molecules that were mapped in the proteome of *Pseudomonas* sp. M1 (Supporting Information Table A) decreased significantly in cells grown in pyruvate medium supplemented with β-myrcene or in β-myrcene-medium. These proteins include the major *Pseudomona*s porin OprF whose primary function is to stabilize the cell envelope structure through the interaction with peptidoglycan [26] and the outer membrane OprD. The lack or low abundance in OprF and OprD is known to contribute to the high levels of intrinsic resistance to toxic agents [26]. Several periplasmic-binding proteins putatively involved in the transport of different solutes are also among the down-regulated proteins. In contrast, the abundance of one porin (spot 12) was significantly increased in cells grown in β-myrcene-media. Although the specific roles of the proteins included in this functional group (porins and periplasmic-binding proteins) are diverse, apparently they also play important structural roles, contributing to cell wall and membrane organization and integrity. The alteration of cell wall and membrane proteins may reflect an adaptive mechanism to the presence of the highly hydrophobic compound β-myrcene in the growth medium, as described for the alteration of murein and muropeptides composition caused by exposure to oregano essential oil in *E. coli* [27]. Furthermore, an increased abundance of the periplasmic β-glucosidase (BglX, spot 84) was also registered in *Pseudomonas* sp. M1 cells grown in β-myrcene-containing media. BglX was implicated in the hydrolysis of anhydro-muropeptides [28], which suggest a role in the alteration of cell wall muropeptides in cells grown in β-myrcene.

3.2.4 β-Myrcene-dependent increased abundance of chaperones and other proteins involved in protein modification and turnover

In general, molecular chaperones assist in the folding of newly synthesized proteins and in protein targeting to membranes [29], and prevent the misfolding and irreversible aggregation of proteins under both normal and stress conditions [30]. The relative abundance of the chaperone proteins GroES, GroEL, DnaK, HtpG and ClpB was found to increase (in the range from 1.5- to 2.0-fold) in cells of *Pseudomonas* sp. M1 grown in β-myrcene-containing media compared with cells grown in pyruvate mineral medium (Table 2). Therefore, it suggests that these molecular chaperones are required for specific processes occurring in

Table 2. Relative alteration (above 1.5-fold or below 0.6) of the content of proteins other than the β-myrcene catabolic enzymes when cells of *Pseudomonas sp.* M1 were grown in β-myrcene supplemented media (Myr, PM1 or PM3) compared with cells grown in mineral medium with pyruvate as sole carbon source (Pyr)

Spot	Protein	Fold change[a)]			ANOVA (*p*)
		Myr/Pyr	PM1/Pyr	PM3/Pyr	
Amino acid metabolism					
32	D-3-phosphoglycerate dehydrogenase, SerA	2.2	2.5	2.5	4.02E-08
81	Dihydroxy-acid dehydratase, IlvD	1.8	2.0	2.1	0.016338
89	Acetolactate synthase III large subunit, IlvI	1.3	1.7	1.6	5.65E−05
97	Serine hydroxymethyltransferase, GlyA	1.5	1.4	1.6	0.029309
Energy metabolism					
14	Citrate synthase, GltA	0.4	0.6	0.4	0.000242
71	Pyruvate dehydrogenase complex, dehydrogenase (E1)component, AceE	0.4	0.6	0.4	1.79E−09
75	Pyruvate carboxylase subunit B	1.5	1.5	1.5	0.001549
78	Phosphoenolpyruvate carboxykinase, PckA	2.1	1.5	4.2	1.44E−15
79	ATP synthase subunit A, AtpA	1.0	1.5	1.4	9.17E−05
Posttranslational modification, protein turnover and chaperones					
22	Co-chaperonin GroES	1.5	1.2	1.4	1.04E−08
31	Lon protease	1.5	1.2	1.5	0.040031
64	Chaperone, GroEL	1.8	1.1	1.8	3.83E−08
66	Chaperone DnaK	1.9	1.6	1.9	5.59E−05
68	Heat shock protein 90, HtpG	1.9	1.3	1.8	1.20E−05
69	ATP-dependent heat shock protein, ClpB	1.8	1.5	2.1	1.37E−05
70	ATP-dependent heat shock protein, ClpB	1.9	1.1	1.3	1.61E−09
99	Serine protease MucD	1.5	1.9	1.6	2.96E−06
Transport of small molecules					
12	Porin	2.5	1.0	0.9	5.15E−09
13	ABC amino acid transporter	0.4	0.7	0.5	7.71E−09
17	Periplasmic-binding protein	0.5	0.6	0.6	0.033086
18	Peptide ABC transporter	0.6	0.8	0.6	1.09E−08
38	Outer membrane porin	0.4	0.6	0.4	0.009243
51	ABC transporter, periplasmic-binding protein	0.6	0.7	0.8	8.47E−09
56	Major porin and structural outer membrane porin	0.2	0.3	0.3	1.04E−10
Other functions					
44	Universal stress protein family	0.6	0.8	1.2	0.000202
84	Periplasmic β-glucosidase, BglX	11.9	8.7	7.5	0.013655

Myr, β-myrcene grown cells; Pyr, pyruvate grown cells; PM1, cells grown in pyruvate medium and β-myrcene for 1 h; PM3, cells grown in pyruvate medium and β-myrcene for 3 h.

a) Fold change was calculated as: [(spot-normalized volume in samples grown inβ-myrcene-containing media)/(spot-normalized volume in reference sample)]/[(spot-normalized volume in pyruvate-grown cells)/(spot-normalized volume in reference sample)]. Reference sample (also known as internal standard) was obtained by mixing equal amounts of proteins from all tested conditions. Each spot-normalized volume corresponds to values obtained from four 2-DE gels analysis.

cells cultivated in the presence of β-myrcene. Hypothetically, these chaperones may be required for the proper folding of the β-myrcene catabolic enzymes and modulators of their activity since their expression levels are much higher in cells grown in β-myrcene even when pyruvate is also present in the growth medium (Table 2). In addition, it is also possible that the presence of β-myrcene or the accumulation of (toxic) metabolites resulting from its catabolism may lead to the induction of different molecular defensive mechanisms in which the chaperones play an important role. Supporting this hypothesis, the Lon protease and the serine protease MucD, which are up-regulated under stress conditions [31], also exhibited a higher abundance in cells grown in β-myrcene-supplemented media (Table 2). These proteases degrade misfolded proteins, thus being indirectly involved in the prevention of protein aggregation [30].

3.2.5 β-Myrcene-induced alteration of the content of other proteins

The increased abundance of proteins involved in the biosynthesis of specific aminoacids was also registered in cells grown in β-myrcene-containing media (Table 2). This

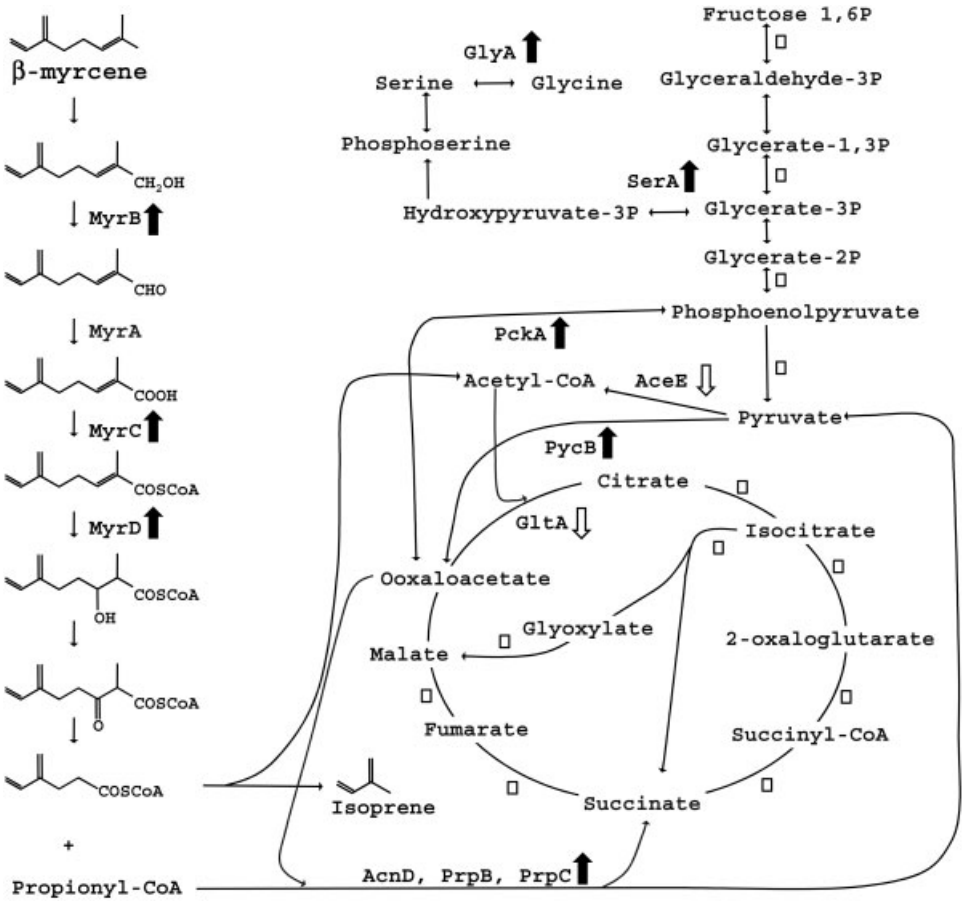

Figure 5. Schematic representation of β-myrcene channeling into central metabolism, evidencing the effect of the presence of β-myrcene in the growth medium in the relative amount of the different enzymes of the central energetic metabolism and on the serine/glycine metabolism in *Pseudomonas* sp. M1 cells grown in pyruvate medium with β-myrcene or in β-myrcene-mineral medium. Black or white arrows indicate increased or decreased abundance, respectively, of the corresponding enzyme in β-myrcene-containing media. A similar expression level of a specific enzyme in the presence or in the absence of β-myrcene is represented by squares.

is the case of dihydroxy-acid dehydratase (IlvD) and acetolactate synthase III large subunit (IlvI) involved in valine, leucine and isoleucine biosynthesis, and of the proteins D-3-phosphoglycerate dehydrogenase (SerA) and serine hydroxymethyltransferase (GlyA), involved in serine and glycine biosynthesis.

4 Concluding remarks

The microbial biotransformation of terpenoids is expected to lead to the production of chemicals with novel enhanced pharmacological, organoleptic and agrochemical properties [32]. Despite the well-described properties of β-myrcene (as referred Section 1), little attention has been given to the microbial biotransformation of this terpene. Therefore, this study aims to expand the necessary knowledge base to capitalize on *Pseudomonas* sp. M1 β-myrcene-related machinery to develop biotechnological tools for the production of β-myrcene derivatives (or chemically related substrates) with potential application in different industrially related processes.

In this study, it was demonstrated, for the first time, that the expression of β-myrcene catabolic enzyme machinery is strongly induced in *Pseudomonas* sp. M1 when β-myrcene is the sole C-source in the growth medium. When pyruvate is present, as an alternative C-source in the growth medium, the β-myrcene-dependent induction of the catabolic pathway was also observed, although at a lower level. The β-myrcene catabolic pathway involves proteins leading to the production of propionyl-CoA and 4-methylene-5-hexenoyl-CoA. This later metabolite is, presumably, converted into acetyl-CoA and isoprene, a volatile metabolite which is known to be excreted by *Pseudomonas* [33]. This β-myrcene catabolic pathway requires an adjustment of the central energetic metabolic flux of M1 leading to the production of oxaloacetate that is required for the proper channeling of β-myrcene-derived metabolites into the central metabolism. Moreover, β-myrcene-efficient catabolism may be dependent on cell's envelope reorganization of M1 strain, as suggested by the registered concerted alteration of the content of different outer membrane and periplasmic-binding proteins.

Based on the phenotypic analysis of the *acnD* knockout mutant M1-2 and on results from the expression proteomic analysis carried out, it is suggested that the β-myrcene catabolic intermediate propionyl-CoA is channeled into the central energetic metabolism *via* the 2-methylcitrate cycle [23] through the action of PprB, PprC and AcnD proteins. It

is hypothesized that M1-2 mutant accumulates 2-methylcitrate when cells are grown in the presence of β-myrcene. This intermediate metabolite is a potent inhibitor of enzymes involved in key biological processes (*e.g.* aconitase and citrate synthase) and therefore is of potential biotechnological interest to develop new drugs aiming selective cell growth inhibition/blockage [23].

Several protein spots whose relative abundance was strongly induced by β-myrcene could not be identified. Examples of hydrophobic substrate uptake transporters in *Pseudomonas* strains have been reported, as it is the case of XylN porin involved in the uptake of m-xylene [34], and we hypothesize that among those unknown proteins an uptake system for β-myrcene may be present, having in mind the registered $_{max}$ for M1 cells growing in β-myrcene as sole C-source. The existence of a XylN-like uptake system for β-myrcene could facilitate β-myrcene initial oxidation, especially if the hydroxylase which catalyses this oxidation is membrane associated, as reported for other aliphatic hydroxylases [35].

This work has provided a number of new insights into the response registered during β-myrcene catabolism by *Pseudomonas* sp. M1, the only described bacterium capable of mineralizing this terpene. However, the enzyme(s) responsible for the initial oxidation of β-myrcene remain unknown, an issue that will be addressed in a near future. Further studies are also required to understand the impact of β-myrcene at the level of full membrane proteome and lipid composition as well as the mechanisms, which are used by M1 to sense the presence of this terpene.

The authors have declared no conflict of interest.

5 References

[1] Schwab, W., Metabolome diversity: too few genes, too many metabolites? *Phytochemistry* 2003, *62*, 837–849.

[2] Pichersky, E., Gershenzon, J., The formation and function of plant volatiles: perfumes for pollinator attraction and defense. *Curr. Opin. Plant Biol.* 2002, *5*, 237–243.

[3] Aharoni, A., Jongsma, M. A., Bouwmeester, H. J., Volatile science? Metabolic engineering of terpenoids in plants. *Trends Plant Sci.* 2005, *10*, 594–602.

[4] Galili, G., Galili, S., Lewinsohn, E., Tadmor, Y., Genetic, molecular, and genomic approaches to improve the value of plant foods and feeds. *Crit. Rev. Plant Sci.* 2002, *21*, 167–204.

[5] Hansen, P. L., Pommer, K., Malmgren, B., Hansen, O. C., Poulsen, M., *Survey of Chemical Substances in Consumer Products*, Danish Environmental Protection Agency, Denmark 2006, p. 66.

[6] Kauderer, B., Zamith, H., Paumgartten, F. J. R., Speit, G., Evaluation of the mutagenicity of beta-myrcene in mammalian-cells *in vitro*. *Environ. Mol. Mutagen.* 1991, *18*, 28–34.

[7] Lorenzetti, B. B., Souza, G. E. P., Sarti, S. J., Santos, D., Ferreira, S. H., Myrcene mimics the peripheral analgesic activity of lemongrass tea. *J. Ethnopharmacol.* 1991, *34*, 43–48.

[8] Matsuura, R., Ukeda, H., Sawamura, M., Tyrosinase inhibitory activity of citrus essential oils. *J. Agric. Food Chem.* 2006, *54*, 2309–2313.

[9] Iurescia, S., Marconi, A. M., Tofani, D., Gambacorta, A. *et al.*, Identification and sequencing of beta-myrcene catabolism genes from *Pseudomonas* sp. strain M1. *Appl. Environ. Microbiol.* 1999, *65*, 2871–2876.

[10] Santos, P. M., Mignogna, G., Heipieper, H. J., Zennaro, E., Occurrence and properties of glutathione *S*-transferases in phenol-degrading *Pseudomonas* strains. *Res. Microbiol.* 2002, *153*, 89–98.

[11] Santos, P. M., Roma, V., Benndorf, D., Von Bergen, M. *et al.*, Mechanistic insights into the global response to phenol in the phenol-biodegrading strain *Pseudomonas* sp. M1 revealed by quantitative proteomics. *Omics – J. Integr. Biol.* 2007, *11*, 233–251.

[12] Santos, P. M., Sa-Correia, I., Characterization of the unique organization and co-regulation of a gene cluster required for phenol and benzene catabolism in *Pseudomonas* sp. M1. *J. Biotechnol.* 2007, *131*, 371–378.

[13] Narushima, H., Omori, T., Minoda, Y., Microbial oxidation of beta-myrcene. *Adv. Biotechnol. III: Ferment. Prod.* 1981, *3*, 525–531.

[14] Hartmans, S., Smits, J. P., Vanderwerf, M. J., Volkering, F., Debont, J. A. M., Metabolism of styrene oxide and 2-phenylethanol in the styrene-degrading *Xanthobacter* Strain 124x. *Appl. Environ. Microbiol.* 1989, *55*, 2850–2855.

[15] Herrero, M., Delorenzo, V., Timmis, K. N., Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. *J. Bacteriol.* 1990, *172*, 6557–6567.

[16] Sambrook, J., Russell, D. W., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY 2001.

[17] Santos, P. M., Benndorf, D., Sa-Correia, I., Insights into *Pseudomonas putida* KT2440 response to phenol-induced stress by quantitative proteomics. *Proteomics* 2004, *4*, 2640–2652.

[18] Shevchenko, A., Tomas, H., Havlis, J., Olsen, J. V., Mann, M., In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat. Protoc.* 2006, *1*, 2856–2860.

[19] Suckau, D., Resemann, A., Schuerenberg, M., Hufnagel, P. *et al.*, A novel MALDI LIFT-TOF/TOF mass spectrometer for proteomics. *Anal. Bioanal. Chem.* 2003, *376*, 952–965.

[20] Perkins, D. N., Pappin, D. J., Creasy, D. M., Cottrell, J. S., Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* 1999, *20*, 3551–3567.

[21] Reva, O. N., Weinel, C., Weinel, M., Bohm, K. *et al.*, Functional genomics of stress response in *Pseudomonas putida* KT2440. *J. Bacteriol.* 2006, *188*, 4079–4092.

[22] Solé, M., Rius, N., Lorén, J. G., Rapid extracellular acidification induced by glucose metabolism in non-proliferating cells of *Serratia marcescens*. *Int. Microbiol.* 2000, *3*, 39–43.

[23] Ewering, C., Heuser, F., Benolken, J. K., Bramer, C., Steinbuchel, A., Metabolic engineering of strains of *Ralstonia eutropha* and *Pseudomonas putida* for biotechnological production of 2-methylcitric acid. *Metab. Eng.* 2006, *8*, 587–602.

[24] Sauer, U., Eikmanns, B. J., The PEP-pyruvate-oxaloacetate node as the switch point for carbon flux distribution in bacteria. *FEMS Microbiol. Rev.* 2005, *29*, 765–794.

[25] Turina, A. V., Nolan, M. V., Zygadlo, J. A., Perillo, M. A., Natural terpenes: self-assembly and membrane partitioning. *Biophys. Chem.* 2006, *122*, 101–113.

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

[27] Caillet, S., Shareck, F., Lacroix, M., Effect of gamma radiation and oregano essential oil on murein and ATP concentration of *Escherichia coli* O157:H7. *J. Food Prot.* 2005, *68*, 2571–2579.

[28] Votsch, W., Templin, M. F., Characterization of a beta-*N*-acetylglucosaminidase of *Escherichia coli* and elucidation of its role in muropeptide recycling and beta-lactamase induction. *J. Biol. Chem.* 2000, *275*, 39032–39038.

[29] Herman, C., D'Ari, R., Proteolysis and chaperones: the destruction/reconstruction dilemma. *Curr. Opin. Microbiol.* 1998, *1*, 204–209.

[30] Winter, J., Jakob, U., Beyond transcription – new mechanisms for the regulation of molecular chaperones. *Crit. Rev. Biochem. Mol. Biol.* 2004, *39*, 297–317.

[31] Yorgey, P., Rahme, L. G., Tan, M. W., Ausubel, F. M., The roles of mucD and alginate in the virulence of *Pseudomonas aeruginosa* in plants, nematodes and mice. *Mol. Microbiol.* 2001, *41*, 1063–1076.

[32] Klein-Marcuschamer, D., Ajikumar, P. K., Stephanopoulos, G., Engineering microbial cell factories for biosynthesis of isoprenoid molecules: beyond lycopene. *Trends Biotechnol.* 2007, *25*, 417–424.

[33] Scholler, C., Molin, S., Wilkins, K., Volatile metabolites from some gram-negative bacteria. *Chemosphere* 1997, *35*, 1487–1495.

[34] Kasai, Y., Inoue, J., Harayama, S., The TOL plasmid pWW0 xylN gene product from *Pseudomonas putida* is involved in m-xylene uptake. *J. Bacteriol.* 2001, *183*, 6662–6666.

[35] van Beilen, J. B., Penninga, D., Witholt, B., Topology of the membrane-bound alkane hydroxylase of *Pseudomonas oleovorans*. *J. Biol. Chem.* 1992, *267*, 9194–9201.

