Physiology and transcriptome of the polycyclic aromatic hydrocarbon-degrading *Sphingomonas* sp. LH128 after long-term starvation

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The survival, physiology and gene expression profile of the phenanthrene-degrading *Sphingomonas* sp. LH128 was examined after an extended period of complete nutrient starvation and compared with a non-starved population that had been harvested in exponential phase. After 6 months of starvation in an isotonic solution, only 5% of the initial population formed culturable cells. Microscopic observation of GFP fluorescent cells, however, suggested that a larger fraction of cells (up to 80%) were still alive and apparently had entered a viable but non-culturable (VBNC) state. The strain displayed several cellular and genetic adaptive strategies to survive long-term starvation. Flow cytometry, microscopic observation and fatty acid methyl ester (FAME) analysis showed a reduction in cell size, a change in cell shape and an increase in the degree of membrane fatty acid saturation. Transcriptome analysis showed decreased expression of genes involved in ribosomal protein biosynthesis, chromosomal replication, cell division and aromatic catabolism, increased expression of genes involved in regulation of gene expression and efflux systems, genetic translocations, and degradation of rRNA and fatty acids. Those phenotypic and transcriptomic changes were not observed after 4 h of starvation. Despite the starvation situation, the polycyclic aromatic hydrocarbon (PAH) catabolic activity was immediate upon exposure to phenanthrene. We conclude that a large fraction of cells maintain viability after an extended period of starvation apparently due to tuning the expression of a wide variety of cellular processes. Due to these survival attributes, bacteria of the genus *Sphingomonas*, like strain LH128, could be considered as suitable targets for use in remediation of nutrient-poor PAH-contaminated environments.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are widespread environmental contaminants in soil and sediments (Lamoureux & Brownawell, 1999). Microbial degradation is considered to be a major process that contributes to the removal of PAHs in soil (Fiala *et al.*, 1999). One of the important bacterial genera implicated in the biodegradation of PAHs is *Sphingomonas*. Members of the genus *Sphingomonas* are strictly aerobic, Gram-negative, rod shaped and chemoheterotrophic bacteria that are widely distributed in soil, water and sediments (Bastiaens *et al.*, 2000; Leys *et al.*, 2004). They have been isolated as aerobic organic xenobiotic- and PAH-degraders and, recently, their association with actual PAH biodegradation in soil has been shown by nucleic acid- and lipid-based stable-isotope probing (Johnsen *et al.*, 2002; Jones *et al.*, 2011).

In the environment, bacteria are frequently challenged by changes in nutrient availability or other physico-chemical...
and biological stress factors (Givskov et al., 1994; Vorob’eva, 2004). Soil environments are often characterized by scarce nutrient supply which influences microbial survival and activity (Givskov et al., 1994; Morita, 1988; Vesteråås et al., 2011). However, many bacteria display adaptive strategies that allow them to survive starvation stress and to resuscitate growth and activity when conditions are favourable. These responses to starvation stress include reduced cytoplasmic molecular crowding, condensation of chromosomal DNA, degradation of cellular components (RNAs, proteins and fatty acids), decreased synthesis of ribosomal proteins, and expression of proteins with high nutrient affinity (Britos et al., 2012). Upon starvation, bacteria first express high affinity systems for uptake and/or metabolism in order to scavenge and use residual nutrients present at limited concentrations in the environment. Examples of such scavenging regulons are the PstSCAB system (ABC-type phosphate-specific transport system) for uptake of inorganic phosphate (Ishige et al., 1991) and the NtrB/NtrC system (two-component regulatory system) for scavenging of nitrogen (Matin, 1991; Peterson et al., 2005). If residual nutrients are exhausted, the viability of the cell is maintained by utilization of stored nutrient sources (Zundel et al., 2009), the use of nutrients originating from dead cells (Herbert & Foster, 2001; Kolter et al., 1993) and/or arrest of cell growth and cell division (Britos et al., 2011; Zundel et al., 2009). Most of the studies that have examined bacterial starvation stress response have addressed either carbon, nitrogen or phosphorus starvation for short durations of a few weeks or months (Betts et al., 2002; Kenyon et al., 2002; Mandel & Silhavy, 2005). Moreover, most of these studies focused on pathogenic bacteria with a few exceptions that address organic-pollutant-degrading soil bacteria like Pseudomonas putida KT2442 (Givskov et al., 1994). As such, not much is known about the adaptive response of PAH-degrading Sphingomonas during long-term nutrient starvation. Information about the survival strategy and adaptive mechanism of Sphingomonas during nutrient starvation will help us understand how these bacteria survive in PAH-contaminated soil with limited nutrient availability, which is of interest for remediation of such environments.

In this study, the survival, physiology, morphology and genome-wide gene expression profile of the phenanthrene-degrading Sphingomonas sp. LH128 were compared before (non-starved cells) and after an extended period of 6 months of complete nutrient starvation (starved cells) in an isotonic solution (0.01 M MgSO₄) in order to acquire information about the strains’ capacities to cope with long-term nutrient deprivation and the mechanisms involved. The non-starved starting LH128 cell suspension was harvested from a culture growing in the exponential phase. To examine whether observed differences were due to direct cell adaptation to the incubation conditions in 0.01 M MgSO₄, identical analyses were performed after 4 h of suspension in 0.01 M MgSO₄, a time period that is sufficient for a change in mRNA expression profile in bacteria (Fida et al., 2012; Golding et al., 2005). Survival was monitored by plating and microscopic counting while the morphology and physiology were examined by flow cytometry, membrane fatty acid methyl ester (FAME) analysis and analysing the phenanthrene mineralization activity. Transcriptomic analysis was performed by whole-genome microarray analysis.

METHODS

Bacterial strain and growth conditions. A GFP-labelled variant of Sphingomonas sp. LH128 (Bastiens et al., 2000; Wouters et al., 2010) was used. Based on its 16S rRNA gene sequence (accession number KCS99553), strain LH128 is phylogenetically most closely related to Sphingomonas subarctica, which belongs to the Novosphingobium branch of the genus Sphingomonas (Takeuchi et al., 2001). The strain was routinely grown at 25 °C in phosphate buffered minimal medium (MM) containing 2 g glucose l⁻¹. MM was prepared as described previously (Uyttebroek et al., 2006).

Experimental set-up. Triplicate cultures of strain LH128 were grown at 20 °C until reaching the exponential phase (OD₆₀₀ of 0.5) in 50 ml MM containing 2 g glucose l⁻¹. Ten millilitres of each culture, containing around 1.4 × 10⁶ c.f.u. ml⁻¹, was centrifuged at 5000 g for 10 min in a Becton Dickinson centrifuge, washed twice with 0.01 M MgSO₄ and resuspended in 10 ml 0.01 M MgSO₄ as an isotonic solution. Three millilitres of each washed culture was immediately used for flow cytometry analysis, analysis of ¹⁴C-phenanthrene mineralization kinetics, whole-cell fatty acid analysis, plating and total RNA extraction for microarray analysis. These immediately analysed suspensions represented the non-starved cells since they were harvested in the exponential phase of growth and were the origin of the starved cells. The remaining of the suspensions were kept at 20 °C for 6 months without shaking, during which the number of c.f.u. was determined at different time points. After 6 months of incubation (referred to as 6 month starved cells), triplicate suspensions were harvested to perform the same analyses as done for the non-starved cells. In a separate experiment, triplicate suspensions of LH128 cells in 0.01 M MgSO₄, identically grown and washed as reported above, were used for total RNA extraction for microarray analysis after 4 h of incubation (referred to as 4 h starved cells) at 20 °C. In a third identically set-up experiment, GFP fluorescent cells were counted at different time points during long-term starvation.

Determination of c.f.u. and microscopic counting. Counting of c.f.u. was carried out as follows. The cell suspension was mixed by vortexing and 200 μl was taken twice from each triplicate suspension. The suspensions were serially diluted 10-fold in 0.01 M MgSO₄ in 96-well microtitre plates, from which 5 μl samples were spotted three times on square MM agar plates (12 × 12 × 1.7 cm) containing 2 g glucose l⁻¹. The plates were incubated at 25 °C and colonies that appeared after 3 days were counted. GFP fluorescent cells were counted after vortexing in a Becton Dickinson chamber by means of an epifluorescence microscope (BX51) coupled with a digital camera (Olympus) and equipped with filter set U-M41001 composed of a 461–500 nm excitation filter and a 521–560 barrier filter.

Flow cytometry analysis. Flow cytometry analysis was performed using an Influx apparatus (BD Biosciences). Half a millilitre of the cell suspension from the three replicate tubes was washed by centrifugation at 3000 g for 10 min and the cells were resuspended in 1 ml 0.01 M MgSO₄. The cell suspension was injected and data were...
acquired until approximately 50,000 events were counted. GFP fluorescent cells were excited using a 488 nm band pass filter and collected through the FL1 detector channel using a 530 nm band pass filter. Analysis of data was done with FlowJo version 7.6 software (Tree Star). The background noise was determined with 0.01 M MgSO₄ without cells.

**FAME analysis.** Whole-cell fatty acids were extracted from 1 ml of each triplicate cell suspension, converted into FAMEs, and analysed as described (Fida et al., 2012). Briefly, the cells were centrifuged at 3000 g for 10 min in a Becton Dickinson centrifuge and resuspended in 0.5 ml water, after which the fatty acids were extracted by means of methanol/chloroform as described by Bligh & Dyer (1959). After conversion of the fatty acids into FAMEs, the FAMEs were identified and quantified by GC-quadrupole MS. The degree of saturation of the membrane fatty acids was quantified as the sum of the relative proportions of palmitoleic acid (16 : 1) and stearic acid (18 : 0) divided by the sum of the relative proportions of palmitic acid (16 : 0) and stearic acid (18 : 0) divided by the sum of the relative proportions of palmitoleic acid (16 : 1) and cis-vaccenic acid (18 : 1Δ11cis). These four fatty acids together compose about 95% of the membrane phospholipid fatty acids in members of the genus *Sphingomonas* (Yabuchi et al., 2001).

**14C-phenanthrene mineralization assay.** Phenanthrene mineralization was examined for all three triplicate suspensions as described previously (Fida et al., 2012) by measuring the production of 14CO₂ from dissolved [9,14C]-labelled phenanthrene (Sigma; 13.1 mCi (485 MBq) mmol⁻¹, dissolved in methanol; radiochemical purity 98.9%). The assay vials were inoculated with 0.5 ml cell suspensions that had been adjusted with 0.01 M MgSO₄ in such a way that they contained 8.7 × 10¹⁶ GFP fluorescent cells in such a way that they contained 8.7 × 10¹⁶ GFP fluorescent cells. The reaction was performed for 2 h at 55 °C. The 'Minimum Information About a Microarray Experiment (MIAME)' procedure (Brazma et al., 2001) was followed for microarray analysis. The microarray data were deposited in NCBI’s Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO series accession numbers GSE44310 and GSE41814 for 4 h starvation and GSE41814 for 6 month starvation samples.

**RNA extraction and transcriptome analysis.** RNA was immediately extracted from 1 ml of each of the triplicate cell suspensions in 0.01 M MgSO₄ using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. The purity and quality of the RNA was determined as described previously (Fida et al., 2012). Design of microarray probes, cDNA labelling, array hybridization, data processing and analysis were performed as described previously (Fida et al., 2012) with minor changes in the nucleic acid labelling procedure, i.e. the labelling reaction was performed using 4 μg of RNA and Superscript III reverse transcriptase (Invitrogen) while the heating reaction was performed for 2 h at 55 °C. The 'Minimum Information About a Microarray Experiment (MIAME)' procedure (Brazma et al., 2001) was followed for microarray analysis. The microarray data were deposited in NCBI’s Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO series accession numbers GSE44310 for 4 h starvation and GSE41814 for 6 month starvation samples.

**Statistical analysis.** Unless otherwise indicated, a two-tailed Student's t-test with a P value cut-off of 0.05 was used to test significance of differences between the control cell suspensions and the starved cell suspensions using SPSS version 11.5 software.

**RESULTS**

**Determination of c.f.u. and microscopic counting**

Numbers of c.f.u. were determined at six time points: after 4 h, 10 days, 30 days, 90 days, 120 days and 180 days of incubation. Counting showed that there was no difference in c.f.u. numbers between the non-starved cells and cells that were incubated for 4 h. However, there was a slight reduction in c.f.u. from 1.4 ± 2 × 10⁹ to 8 ± 3.8 × 10⁸ c.f.u. ml⁻¹ after 10 days of incubation (Fig. 1). Upon extended duration of starvation (6 months), the c.f.u. numbers declined to about 7 ± 4 × 10⁷ ml⁻¹, i.e. about 5% of the population was culturable. Microscopic observations showed that, in contrast with the initial suspension, after 6 months of starvation the cells aggregated and formed clusters of two to ten cells, which explains partially the reduction in c.f.u. numbers. Since GFP has been proposed as a marker for cell viability (Lowder et al., 2000), GFP fluorescent cells were counted by means of epifluorescence microscopy in a separate but identically set-up experiment. No reduction in fluorescent cell number was observed after 4 h and 10 days of starvation as compared to the initial non-starved cell suspension while c.f.u. numbers had slightly decreased at day 10 (data not shown). After 7 months, about 80% of the cells still showed GFP while only 2.2% formed c.f.u. (data not shown).

**Morphological and physiological response to starvation**

Flow cytometry analysis indicated cell size reduction after 6 months of starvation as compared to freshly prepared non-starved cells (Fig. 2). In addition, microscopic observation of GFP fluorescent LH128 cells showed that the majority of the cells changed their shape from a rod shape (Fig. 3a) to a more round shape (Fig. 3b). No changes in cell morphology were observed after 4 h of incubation in 0.01 M MgSO₄ (data not shown). FAME analysis showed that the degree of cell membrane fatty acid saturation was significantly increased (P=0.003) in the 6 month starved cells as compared with the non-starved cells (Fig. 4).

**Fig. 1.** Dynamics of c.f.u. numbers of *Sphingomonas* sp. LH128 during long-term incubation in 0.01 mM MgSO₄. The numbers determined at time 0 and 4 h were identical and are therefore indistinguishable from each other on the graph. Values are mean values of three replicates. The error bars indicate the standard deviation.
Phenanthrene mineralization activity

Cells starved for 6 months and transferred to a mineralization medium containing 14C-phenanthrene showed phenanthrene mineralization kinetics that were not significantly different from those displayed by the non-starved cells regarding lag time or mineralization rate. For both suspensions, about 21% of the phenanthrene was mineralized after 24 h of incubation (Fig. 5).

Gene expression analysis

Total RNA yields of 5.5 ± 0.5, 5.53 ± 0.8 and 3.5 ± 0.9 µg ml⁻¹ were obtained, respectively, from the non-starved cells and the 4 h and 6 month starved cells. Microarray analysis was used to determine the global transcriptomic response of 4 h and 6 month starved LH128 cells as compared with the non-starved suspension. Both hierarchical clustering analysis and principal component analysis (PCA) showed clustering of the replicates for both the non-starved cell suspensions as well as starved (4 h and 6 months) cell suspensions with distinct clusters for each suspension (data not shown). Of all targeted putative protein coding ORFs, only 0.33% (23/7033) of the genes were differentially expressed in the 4 h starved cell suspension while 42% (2973/7033) of the genes were compared with the non-starved suspension.

Fig. 2. Cell size distribution of starved Sphingomonas sp. LH128 (dotted line) as compared to non-starved cells in exponential growth phase (solid line). Values on the y-axis are ‘Percentage of maximum’, in which ‘maximum’ refers to the cells that showed the most frequently encountered forward scatter (FSC) value.

Fig. 3. Microscopic observations of non-starved Sphingomonas sp. LH128 cells (a) and after 7 months of starvation (b). Images were captured at ×1000 magnification. White arrows indicate the normal rod shape of the strain and the red arrow indicates the round-shaped variant.

Fig. 4. Degree of saturation of whole-cell fatty acids of 6 month starved Sphingomonas sp. LH128 cells as compared to non-starved cells. Values shown are the mean of three biological replicates with indicated standard deviation. Asterisk indicates that the value is significantly different from that of the control (P value <0.05).

Fig. 5. 14C-phenanthrene mineralization activity of 6 month starved (black bars) and non-starved (white bars) Sphingomonas sp. LH128 cells. Values are expressed as the percentage of the initial amount of phenanthrene that was converted into CO₂ and are the mean of three biological replicates, each of which was measured three times, with indicated standard deviation (n=9).
differentially expressed in the 6 month starved cell suspension, as compared with the non-starved cell suspension (Table 1). About 70% and 53% of the differentially expressed genes showed increased expression at 4 h and 6 months starvation, respectively, while 30% and 47% showed reduced expression, respectively. Only five of the differentially expressed genes were in common between the suspensions incubated for 4 h and those incubated for 6 months. About 35% and 49% of the differentially expressed genes after 4 h and 6 months of starvation, respectively, encoded hypothetical proteins or proteins with no known function. Differentially expressed genes that could be related to proteins with known function were classified into clusters of orthologous groups (COG) (Tatusov et al., 1997) based on their predicted functions (Fig. 6). Since only 14 genes with known functions were differentially expressed in 4 h starved cells, the COG classification is not included in the figure. Genes encoding putative proteins in the categories of inorganic ion transport and metabolism, lipid transport and metabolism, cell wall/membrane/envelope biogenesis, and recombination/repair comprised the largest number of genes with increased expression after 6 months of starvation. Genes encoding putative proteins in the category of transcription, translation/ribosomal structure and biogenesis, RNA processing and modification, and cell cycle control/cell division/chromosome partitioning represented the largest number of genes with reduced expression. A list of the differentially expressed genes (twofold or more) is presented in Tables S1 and S2 (available in Microbiology Online), respectively, for the 4 h and 6 month starved suspensions. In the following paragraphs, the major differentially expressed putative genes with annotated functions are presented.

Among the genes differentially expressed after 6 months of starvation were genes for coping with osmotic stress, which included the genes encoding homologues of proteins required for biosynthesis of ectoine and trehalose. Differential expression of these genes was not observed in 4 h starved cells. In addition, many of the genes involved in transport of ions and efflux systems across the membrane showed increased expression at 6 months of starvation. These included genes encoding homologues of the major facilitator superfamily (MFS) protein, cation/multidrug efflux pumps (acriflavin resistance protein), drug resistance transporters (arabinose efflux permease), conductance mechanosensitive channel proteins (MSC), Na⁺/H⁺ antiporters, Co/Zn/Cd cation transporters, K⁺ transporters, phosphate transporters or proteins specifying resistance-nodulation-division (RND) efflux systems. Genes encoding a sec-independent translocase protein (TatC), a nitrogen regulatory protein P-II, and a GCN5-related N-acetyltransferase showed increased expression at 4 h but reduced expression after 6 months of starvation.

Long-term starvation stress also resulted in an increased expression of genes involved in the sensor and regulatory cascade of transcription including genes encoding homologues of guanosine tetraphosphate and guanosine pentaphosphate [(p)ppGpp] synthase and gene functions involved in antioxidative stress response such as the genes encoding catalase and peroxiredoxin. This was not the case at 4 h of starvation. Furthermore, the transcriptome data at 6 months of starvation showed increased expression of genes encoding different types of RNases and proteins linked to genetic plasticity and exchange. The latter included genes encoding homologues of more than 30 different transposase types (the majority of them showing more than 10-fold increased expression), and genes encoding homologues of integrase, recombinase, resolvase, ligase, single-stranded DNA-binding protein, conjugal DNA transfer protein, and plasmid replication initiator protein. Genes involved in lipid transport and metabolism, such as genes encoding different groups of acyl-CoA dehydrogenases and enoyl-CoA hydratase/isomerase, showed increased expression at 6 months of starvation. None of these genes was differentially expressed at 4 h of starvation.

The transcriptome data further showed a number of genes with reduced expression at 6 months of starvation. Among them were more than 30 genes encoding the different 30S and 50S ribosomal proteins, none of which was differentially expressed at 4 h of starvation. Also, genes encoding proteins putatively involved in replication, such as the RepA replication protein, the DNA replication initiator protein, DNA polymerase III and the antitoxins YefM and

Table 1. Number of genes differentially expressed between starved Sphingomonas sp. LH128 cells (after 4 h and 6 months of incubation) and non-starved LH128 cells. Values in parentheses indicate the percentage of differentially expressed genes of all targeted protein coding ORFs

<table>
<thead>
<tr>
<th>Duration of incubation</th>
<th>Number of differentially expressed genes</th>
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<td></td>
<td>Increased expression</td>
<td>Reduced expression</td>
<td>Total</td>
</tr>
<tr>
<td>4 h</td>
<td>16 (0.23%)</td>
<td>7 (0.1%)</td>
<td>23 (0.33%)</td>
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<tr>
<td>6 months</td>
<td>1584 (22.5%)</td>
<td>1389 (19.7%)</td>
<td>2973 (42.3%)</td>
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<tr>
<td>4 h and 6 months*</td>
<td>–</td>
<td>5 (0.07%)</td>
<td>5 (0.07%)</td>
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*Genes that were differentially expressed at 4 h as well as 6 months of starvation.
RelB, were among those with reduced expression at 6 months of starvation. The gene encoding RelB is the only gene among this group that also showed reduced expression at 4 h of incubation. Unexpectedly, reduced expression of many of the genes encoding heat-shock proteins and chaperones was observed during long-term starvation stress, with the exception of genes encoding the trigger factor, the heat-shock DnaJ-like protein and the heat-shock protein HtpX. In addition, reduced expression of some of the genes involved in PAH degradation was observed at 6 months of starvation stress, which included the genes specifying the initial ring hydroxylating dioxygenase α-subunit (phnA1f) and β-subunit (phnA2f). Differential expression of catabolic genes was not observed in 4 h starved cells. Furthermore, reduced expression of nearly all the genes involved in cell division and partitioning were observed, which included genes encoding homologues of the cell-division proteins FtsI, FtsQ and FtsZ, and the DNA segregation ATPase FtsK/SpoIIE-related protein. None of these genes was differentially expressed at 4 h of starvation.

**DISCUSSION**

The aim of this study was to examine the survival, physiology, morphology and transcriptome of LH128 cells after long-term starvation in order to acquire knowledge about the strain’s capacity to cope with long-term starvation stress and about the mechanisms involved. Even after 6 months of complete nutrient deprivation, 5% of the LH128 cells were still culturable and hence viable. Long-term survival of bacterial cells in nutrient-limited liquid medium has been previously reported for several
other soil bacteria but all were members of the genus *Pseudomonas*, for example *Pseudomonas fluorescens* R2f after carbon starvation for 72 days (van Overbeek et al., 1995), *P. fluorescens* A506 after complete nutrient starvation for 21 days (Lowder et al., 2000), and *P. putida* KT2442 after nitrogen and carbon starvation for 90 days (Givskov et al., 1994). The number of c.f.u. of LH128 deceased with increasing duration of starvation. The reduction in c.f.u. numbers during exposure to nutrient starvation is partly due to cell aggregation but could also be linked to non-culturality, i.e. entrance of the cells into a viable but non-culturable (VBNC) state, rather than to cell death. Entrance into a VBNC state upon complete nutrient starvation was also shown for *P. fluorescens* A506 (Lowder et al., 2000). As for *P. fluorescens* A506, our conclusion is based on the observations that a high fraction (80% in the case of LH128) of cells still showed GFP fluorescence after 7 months of starvation. It looks unlikely that GFP is stable in dead cells. No exact information exists about the half-life of GFP in bacterial cells but Lowder et al. (2000) showed that dead cells of gfp-tagged *P. fluorescens* A506 lost fluorescence while starved cells and VBNC cells remained fluorescent. In mouse cells, GFP has a half-life of about 26 h (Corish & Tyler-Smith, 1999). The observation that a large fraction of the cells were in a VBNC-like state is also supported by the recorded RNA yields, i.e. yields were only reduced by 30% after 6 months of incubation as compared with the non-starved cells. In dead cells, the mRNA is expected to be rapidly degraded by enzymes such as RNases (Anderson & Dumnan, 2009; Deutscher, 2003, 2006; Kushner, 2002; Selinger et al., 2003). The half-life of mRNA in, for instance, *Escherichia coli* is around 6.8 min (Selinger et al., 2003).

Strain LH128 showed several morphological changes after long-term starvation such as a reduction in cell size and change in cell shape, which is consistent with other organisms including *P. fluorescens* R2f (van Overbeek et al., 1995), *P. fluorescens* A506 (Lowder et al., 2000) and the multiple-nutrient-starved marine ultramicrobacterium *Sphingomonas* sp. RB2256 (Eguchi et al., 1996). Those changes were not observed after 4 h of incubation showing that changes in cell morphology are not an immediate response to the suspension in 0.01 M MgSO₄. The physiological advantage of reduction in cell size is not very clear. It is suggested to improve the uptake of scarce nutrients due to the increase in cell surface area to volume ratio (van Overbeek et al., 1995). Also, after inoculation and growth in soil, strain LH128 exhibited a reduced cell size (T. Fida, unpublished result). Change in cell shape or size has been related to reduced expression of genes encoding proteins that determine the size and shape of the cell, such as the rod-shape determining proteins (RodA), cell-division proteins and penicillin-binding proteins (PBP) (Ishino et al., 1986; Noirclerc-Savoye et al., 2003), and increased expression of a gene encoding (p)ppGpp synthase (Atkinson et al., 2011). Corresponding gene orthologues were indeed accordingly differentially expressed in the LH128 cells subjected to long-term starvation, with the exception of rodA. As a physiological adaptation, LH128 cells appear to increase the degree of saturation of membrane fatty acids. An increase in the degree of phospholipid fatty acid saturation reduces cell membrane fluidity and permeability and has been previously reported in a number of bacteria as a response to the presence of solutes or toxic hydrocarbons and to a shift in temperature (Heipieper & de Bont, 1994; Heipieper et al., 2003; Johnson et al., 2011; Kieft et al., 1997; Mrozik et al., 2004; Suutari & Laakso, 1994), but has not yet been related to nutrient starvation. In *Sphingomonas* sp. LH128, an increase in phospholipid fatty acid saturation has been previously observed as a response to chronic NaCl stress in biofilms (Fida et al., 2012) and also after inoculation in soil (T. Fida unpublished data). The change in membrane fatty acid saturation is related to lipid biosynthesis and can only occur in viable cells (Denich et al., 2003; Heipieper & de Bont, 1994; Trevors, 2012). However, no changes were observed in expression levels of genes responsible for the saturation of membrane fatty acids such as fatty acid synthase.

The result from mineralization assays showed that the phenanthrene catabolic activity of the starred cells was similar to that of non-starved cells and as such hardly affected. From another study, we have evidence that the phenanthrene catabolic genes in strain LH128, such as the gene cluster phnA1fA2f which determines the initial attack of phenanthrene, is induced in the presence of phenanthrene (T. Fida, unpublished results). The transcriptomic data showed that expression of the phenanthrene catabolic genes is decreased after 6 months of starvation compared with non-starved cells. This indicated the rapid induction of PAH catabolic genes and rapid resuscitation of PAH catabolic activity upon addition of phenanthrene in starred cells. Rapid mineralization of 2,6-dichlorobenzamide (BAM) after resuscitation has also been reported in carbon- and nitrogen-starved cells of the BAM-degrading *Aminobacter* sp. MSH1 (Sjøholm et al., 2010). Starved cells of *Rhizobium leguminosarum* also rapidly restarted growth when nutrients became available (Thorne & Williams, 1997).

The adaptation to long-term starvation was also observed in the transcriptome, with numbers of differentially expressed genes that were far higher than those previously recorded in LH128 biofilm cells affected by solute stress (Fida et al., 2012) and LH128 cells inoculated in soil (T. Fida, unpublished results). The high number of differentially expressed genes after long-term starvation showed that starvation stress imposes a global shift in gene expression in LH128. Very few (0.33 %) of the genes were differentially expressed after 4 h of incubation under starvation stress showing that the differences in gene expression observed after 6 months is not an immediate response to the changed incubation conditions. This is in contrast with other studies, where LH128 showed a significant change in gene expression after 4 h of salt stress (9 % of the genes).
(Fida et al., 2012) and after 4 h in soil (7% of the genes) (T. Fida, unpublished results). Moreover, no major known starvation-stress-related genes were differentially expressed after 4 h of incubation with the exception of the gene encoding a RelB homologue. Apart from genes that could be related to changes in cell morphology (see above), several other genes whose expression profile could be directly related to starvation stress were identified. One important gene that showed increased expression after long-term starvation was the gene encoding a homologue of the (p)ppGpp synthase gene. (p)ppGpp is known as a master regulator of gene expression in bacteria including soil bacteria such as *E. coli* and *Rhizobium etli* (Traxler et al., 2008; Vercruysse et al., 2011). Starved cells of LH128 also expressed genes that encode RNases, such as RNaseE, which degrades rRNA during starvation stress. In *E. coli*, RNaseE controls global miRNA degradation (Manasherob et al., 2012). RNases also degrade stable RNAs, such as rRNA and tRNA, during starvation stress (Deutscher, 2003; Srivatsan & Wang, 2008). Stable RNAs account for the bulk of cellular RNAs and are used as a potential storehouse of nutrients to help the cell survive during starvation conditions (Chen & Deutscher, 2005). Furthermore, a large number of genes with increased expression are genes encoding putative proteins involved in fatty acid β-oxidation, which might indicate that fatty acids are an important energy and carbon source in LH128 during starvation as reported previously for *Pseudomonas fragi* B-0771 (Sato et al., 1992). Whether these fatty acids are derived from internal sources or dead cells is currently unclear. The use of nutrients originating from dead cells has been reported previously for *Listeria monocytogenes* and *E. coli* (Herbert & Foster, 2001; Kolter et al., 1993). Also, the decreased expression of several genes could be directly related to the long-term starvation situation. For instance, the decreased expression of genes encoding ribosomal proteins during long-term starvation stress is in accordance with other studies that applied different types of stress in a number of bacteria including soil bacteria such as *P. putida* (Givskov et al., 1994) and *R. leguminosarum* (Thorne & Williams, 1997). In addition, the reduced expression of genes involved in replication and cell division is consistent with the growth arrest of cells during starvation stress. Reduced expression of the putative antitoxin homologues YefM and RelB also corroborates reduced cell growth. Both antitoxins participate in growth arrest and enable cell survival for prolonged times during environmental stresses (Liu et al., 2008). Antitoxins have been associated with cell persistence and are transcriptional repressors of toxin biosynthesis genes. When they are downregulated, the toxin is liberated and results in growth arrest (Christensen et al., 2001). In *E. coli* K-12, YefM is encoded by the yoeB–yefM operon (Zhang & Inouye, 2009). yefM is included in a similar operon organization with a yoeB homologue in LH128 with YefB and YefM sharing 48% and 43% amino acid identity, respectively, with the corresponding proteins in *E. coli*. In *E. coli*, RelB is encoded by the relB–relE antitoxin–toxin operon (Li et al., 2009). However, in the draft genome sequence of LH128, relB is located separately downstream of the yoeB–yefM operon in an opposite direction and a homologue of relE is not present. The transcriptome data further showed reduced expression of several genes that demand energy or are not crucial for survival including the phenanthrene catabolic genes. The apparent reduced expression of those genes could be linked to the reduction of biosynthesis of unnecessary proteins as an energy saving mechanism or to the degradation of the corresponding mRNA by RNase activities during starvation. This was not the case in the 4 h starved cells as these cells did not show reduced expression of the corresponding genes or increased expression of RNase-encoding genes.

LH128 cells also appear to cope with long-term starvation stress by increasing the expression of different genes that have been previously primarily related with other stress situations. These include genes involved in antioxidative stress response and genes associated with biosynthesis of osmostropeuctants, such as ectoine and trehalose, as well as various membrane transport proteins. These genes also showed increased expression in *Sphingomonas* sp. strain LH128 during salt stress (Fida et al., 2012) and after inoculation in soil (T. Fida, unpublished results). The relationship between long-term starvation and the increased expression of those genes is not directly clear but is also not unexpected as starved bacterial cells often show increased resistance to a range of environmental stresses including osmotic and oxidative stress compared with growing cultures. This has also been shown for several soil bacteria such as *P. putida* (Givskov et al., 1994), *P. fluorescens* (van Overbeek et al., 1995) and *R. leguminosarum* (Thorne & Williams, 1997). As found for other bacteria, it indicates the existence of a general stress response in *Sphingomonas* sp. LH128 upon long-term starvation. On the other hand, the increased expression of genes related to osmotic stress might be linked to the long-term incubation in the 0.01 M MgSO₄ solution. In addition, as observed as a response to other stresses like salt stress (Fida et al., 2012; Foster, 2007), overexpression of many genes associated with translocation of genetic material was observed, suggesting that genetic rearrangements might occur during growth arrest and long-term starvation in LH128. Reduced expression of many of the genes encoding heat-shock proteins and chaperones was observed during long-term starvation, which was also the case during solute stress of LH128 in biofilms (Fida et al., 2012) and after inoculation in soil (T. Fida, unpublished results). No clear explanation can be given for the reduced expressions since heat-shock proteins prevent the aggregation of stress-denatured proteins in response to temperature stress (Wu et al., 1996). However, this observation is in agreement with gene expression analysis of *E. coli* during...
growth arrest, where decreased expression of several heat-shock proteins was observed (Chang et al., 2002).

In conclusion, LH128 displays several cellular and genetic adaptive strategies to survive under severe nutrient starvation for prolonged periods of time without drastic loss of viability. The starvation stress resulted in changes in shape and a smaller cell size, an increase in the degree of saturation of membrane fatty acids, a decreased expression of gene functions involved in ribosomal protein biosynthesis, chromosomal replication, cell division and aromatic catabolism, an increased expression of gene functions involved in regulation of gene expression and gene translocation, and degradation of rRNA and fatty acids. Due to its ability to survive during long-term nutrient starvation without drastic loss of viability, Sphingomonas sp. LH128 and possibly other members of the genus Sphingomonas are suitable targets for use in remediation of nutrient-poor PAH-contaminated environments.

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REFERENCES


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