RESEARCH ARTICLE

Maintenance of phenol hydroxylase genotypes at high diversity in bioreactors exposed to step increases in phenol loading

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Abstract

To better understand how the composition of bacterial communities changes in response to different environmental conditions, we examined the influence of increasing phenol load on the distribution of the protein-coding functional gene of the largest subunit of phenol hydroxylase (LmPH) and of the 16S rRNA gene in lab-scale activated sludge reactors. LmPH diversity was assessed initially from a total of 124 clone sequences retrieved from two reactors exposed to a low ($0.25\,g\,L^{-1}$) and a high ($2.5\,g\,L^{-1}$) phenol concentration. The quantitative changes in the concentration of the eight detected genotypes accompanied changes in the phenol degradation rates, indicating a community structure–function relationship. Nonmetric dimensional analysis showed that LmPH genotypes and the denaturing gradient gel electrophoresis banding patterns clustered together by phenol concentration, rather than by reactor identity. Seven isolates, representing cultivated strains of each of the observed LmPH genotypes, exhibited a rather narrow range of physiological diversity, in terms of the growth rate and the kinetic parameters of the phenol-degrading activity. We suggest that lab-scale reactors support many ecological niches, which allow the maintenance of a high diversity of ecotypes through varying concentrations of phenol, but the ability of particular strains to become dominant members of the community under the different environmental conditions cannot be predicted easily solely from their phenol-degrading properties.

Introduction

Since phenol became a common constituent of industrial wastewater, it has reached a widespread distribution as a pollutant of water resources. Phenol can be degraded aerobically by a wide variety of microorganisms in pure cultures (Hill & Robinson, 1975; Gonzalez *et al.*, 2001), in mixed consortia (Ambujom, 2001; Chen *et al.*, 2008) and in complex undefined communities, such as activated sludge (Watanabe *et al.*, 1998; Kibret *et al.*, 2000; Zhang *et al.*, 2004; Jiang *et al.*, 2007). Activated sludge processes used to treat phenol-containing wastewaters are commonly subjected to toxic loadings, which affect both the composition and the function of the systems (Watanabe *et al.*, 1996, 1999; Kibret *et al.*, 2000).

Shifts in community species composition or relative species abundances are commonly attributed to functional or adaptive species traits that allow better adaptation or tolerance to the environmental change (Lavorel & Garnier, 2002; Eviner & Chapin, 2003). Although this was already recognized in plant communities (Walker *et al.*, 2006; Bokhorst *et al.*, 2007), the exploration of these fundamental ecological questions in microbial ecosystems is just beginning to be tackled (Prosser *et al.*, 2007). Species traits could be used to predict the response of protozoan diversity to both disturbance and productivity (Haddad *et al.*, 2008). Based on a meta-analysis of the effect of disturbances on microbial communities, Allison & Martiny (2008) concluded that changes due to disturbances may directly affect ecosystem processes and proposed a framework to incorporate microbial community composition into ecosystem process models.

In engineered environments, understanding the way in which bacterial community structure is affected by key

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environmental factors is essential for the rational improvement of treatment process performance and stability (Briones & Raskin, 2003; McMahon *et al.*, 2007). Activated sludge systems usually harbor a large diversity of phenol-degrading bacteria. Phenol hydroxylase catalyzes the conversion of phenol into catechol, the rate-limiting step in the phenol degradation pathway. Of the two different variants of this enzyme found in bacteria, the single-component and the multicomponent types, the latter (mPH) is considered the main phenol hydroxylase enzyme in the environment (Peters *et al.*, 1997). The mechanism, which allows the co-occurrence of bacteria growing on the same substrate, is not fully understood. The use of replicate bioreactors is a convenient means to study the parallel effects of disturbances on microbial composition and function. We have shown previously that limited amounts of phenol in the feed led to a significant increase in the phenol-degrading activity of lab-scale activated sludge, with a concomitant increase in the large subunit of multicomponent phenol hydroxylase (LmPH) genotype diversity (Basile & Erijman, 2008). These results suggested that phenol degradation in the activated sludge depended on the combined activity of a number of redundant species. Given the extent of redundancy, it was reasonable to expect that the systems would respond to changes in environmental conditions by changing the relative abundance of phenol-degrading species. Thus, the initial objective of the present study was to examine the response of the microbial community structure to increasing loads of phenol, by focusing on the protein-coding functional gene LmPH, a proxy for phenol-degrading populations, and of the 16S rRNA gene denaturing gradient gel electrophoresis (DGGE) fingerprints, a surrogate measure for the detectable bacterial diversity. We then hypothesized that the shifts in community structure could be related to the phenol-degrading properties of the intervening species. Therefore, the second goal was to relate the shifts in LmPH genotype diversity to functional traits of representative phenol-degrading isolates.

Materials and methods

Reactors setup and operation

Sludge from the aeration basin of the activated sludge treating wastewater from a petroleum refinery was used as an inoculum to seed four lab-scale sequential batch reactors. The reactors, each with a working volume of 800 mL, were operated at a constant temperature of $28 \pm 1\,^{\circ}C$. A volume of 400 mL of synthetic sewage (K_2HPO_4, 28 mg L^{-1}; $MgSO_4$, 2 mg L^{-1}; NaCl, 7 mg L^{-1}; $CaCl_2 \cdot 2H_2O$, 4 mg L^{-1}; peptone, 160 mg L^{-1}; yeast extract, 110 mg L^{-1}; urea, 30 mg L^{-1}; and phenol, 250 mg L^{-1}, pH 7.3) was supplied over a period of 12 h using a peristaltic pump, with simultaneous aeration. Aeration was continued for an additional 9 h, after which it was interrupted for 2 h for settling. The supernatant was decanted manually at the end of the settling phase. The solid retention time was set at 20 days by wasting daily 5% of the mixed liquor at the end of the aeration period. The wasted sludge was immediately used for activity assays and genetic analysis. During the first 2 months after start up, the four reactors were operated identically. At day 61, the phenol concentration in the feed of two of the reactors was increased from 250 mg L^{-1} to 1.25 g L^{-1}, i.e. the daily phenol amendment changed from 100 to 500 mg of phenol. The concentration of phenol was further increased to 2.5 g L^{-1} at day 82, and finally, to 3.75 g L^{-1} at day 137. After 36 additional days of operation with a phenol load of 3.75 g L^{-1}, phenol amendment was restored to 250 mg L^{-1}. During the entire period, the two control reactors received a phenol concentration of 250 mg L^{-1}.

Measurement of phenol degradation rates

Phenol degradation rates were determined in batch degradation assays. Ten milliliters of wasted sludge from each of the reactors was separately incubated at $28\,^{\circ}C$ in the presence of 20 mg L^{-1} phenol. Phenol concentrations were measured using a standard colorimetric assay, where phenolic compounds react with 4-aminoantipyrine in the presence of potassium ferricyanide to form a colored antipyrine dye (King *et al.*, 1991).

DNA extraction, cloning and sequencing

Extraction of genomic DNA from sludge was performed as described previously (Figuerola & Erijman, 2007). A fragment of the gene encoding the largest subunit of multicomponent phenol hydroxylase (LmPH) was amplified from DNA extracted from both phenol-amended reactors at 108 days of reactor operation, corresponding to a phenol load of 2.5 g L^{-1}. LmPH forward primers U1, U2 and U3 were used in conjunction with the LmPH U reverse primer (Table 1) in three independent PCR reactions. The products were pooled, gel-purified, ligated into the pGEM T-Easy vector (Promega Corp., Madison, WI) and transformed into *Escherichia coli* DH10B cells by electroporation. Plasmid template DNA were prepared using the standard alkaline lysis method. DNA sequencing was performed at the Macrogen sequencing facility (Seoul, Korea).

Phylogenetic analysis

LmPH sequences averaged approximately 600 bp. Nucleotide sequences and the derived amino acid sequences were determined for a total of 65 clones. MEGA software (Tamura *et al.*, 2007) was used to perform phylogenetic analysis. LmPH sequences were aligned to one another, to the 59

Table 1. Oligonucleotides used as PCR primers for LmPH gene quantification

Targets	Forward primers	Reverse primers	bp
L1	CAGATCCACAGCCTGTCC	GGCRTCGTCAAAGAACGA	113
L2	GCGTGGCCTGCCTGATG	GCGTCGTCGAAGAAGCTC	165
L3	TCACTTCCAGACCGAAATG	GCGTCTTCAAAAAATGAC	125
L4	CCACTGCCAGACCGAAAC	CGGCATCTTCAAAGAACG	127
L5	GAGACCCATGCGCTGTCC	GCTCAGCGCATCTTCAAA	119
L6	GAGACSCATGCGYTGAGC	TCATCGCGTCCTCGAAGA	118
L7	ACTGCGTCACTGCCAGAC	TCGCGTCGTCGAAGAAGG	134
L8	GACGAGCTGCGGCATTCG	TCGCATCGTCGAAATACG	132
U1	YCARGSYGARAARGARAAAAARCT	CGRWARCCGCGCCAGAACCA	620
U2	CCAGKCBGARAARGAGCGCAAGYT		
U3	CCAGGSBGARAARGARARGAARCT		
H	U1+U2+U3	GTGGCCATGTCGCCATTGA	473

sequences obtained from DNA extracted from the activated sludge reactors receiving 250 mg L^{-1} of phenol (Basile & Erijman, 2008) and to selected related sequences obtained from the GenBank. Trees were constructed by applying neighbor joining and parsimony. Statistical evaluation of tree topologies was performed using bootstrap analysis with 100 resamplings. Sequence assignment to operation taxonomic units was performed using the furthest-neighbor algorithm of DOTUR program (http://www.plantpath.wisc.edu/fac/joh/dotur.html), using a genetic distance of 0.11 between sequences.

Real-time quantitative PCR (q-PCR)

Quantification of LmPH gene diversity was performed by real-time PCR using a SYBR Green assay on samples collected 20 days after each step increase in phenol loading, on a DNA Engine Opticon 2 Continuous Fluorescence Detection System (Bio-Rad). The sequences of the primers for q-PCR were derived from the LmPH sequences included in the phylogenetic tree in Fig. 1. Specific primer sequences developed in this study are listed in Table 1.

Reactions were carried out using 25-μL reaction mixtures in eight-well reaction strips with optical caps (MJ Research). PCR mixtures contained 1 U Taq Platinum DNA polymerase (Invitrogen), 1 × Taq Platinum buffer, a 0.25 mM concentration of each dNTP, SYBR green I (1 : 50 000; Molecular Probes), dimethyl sulfoxide 5%, primers 0.4 μM and $MgCl_2$ 2 mM. The annealing temperature used for PCR amplification was 59 °C. All primer sets were tested for their specificities using clones belonging to one representative of each nontarget group as negative controls (10^6 copies of competing gene fragments per assay). Standard curves were performed for all the genotype-specific primer pairs.

Real-time PCR analysis was performed as in Basile & Erijman (2008). The percentage of each LmPH subgroup was calculated based on the total copy number of the LmPH gene, determined using a mixture of primers LmPH Uf 1, 2 and 3, and LmPH Ur (Table 1). LmPH universal primers were modified from primers Phe Uf and Phe Ur (Futamata *et al.*, 2001), in order to target additional LmPH nucleotide sequence data obtained from the GenBank (108 sequences for LmPH Uf and 82 sequences for LmPH Ur).

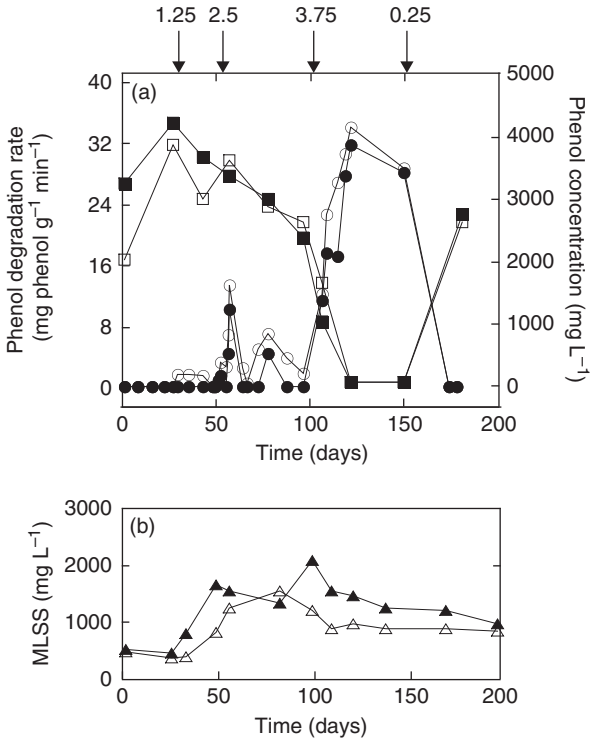


Fig. 1. (a) Phenol degradation rates (■, □), effluent phenol (●, ○), and (b) variation in the mixed liquor suspended solids (MLSS) during aerobic degradation in lab-scale activated sludge reactors. Closed symbols correspond to reactor PH1; open symbols correspond to reactor PH2. The phenol concentration in the feed was increased from 250 mg L^{-1} to 1.25 g L^{-1} on day 61. The concentration of phenol was further increased to 2.5 g L^{-1} on day 82, and finally, to 3.75 g L^{-1} on day 137. On day 173, phenol in the feed was reduced back to 250 mg L^{-1}.

DGGE

PCR amplification of a fragment spanning the V3 region of the 16S rRNA gene from genomic DNA and DGGE were performed as described previously (Lozada *et al.*, 2007). After electrophoresis, gels were soaked for 30 min in SYBR Gold nucleic acid stain (Invitrogen, 1 : 10 000 dilution in TAE; pH 8.0). The stained gels were immediately photographed on a UV transillumination table combined with a camera module and imaging system (Syngene, Cambridge, UK).

Bands of interest were excised using sterile razor blades; the DNA was eluted with 400 μL volume of TE overnight at 4 °C and precipitated with two volumes of ethanol and 100 mM NaCl. The pellet was resuspended in 50 μL of water. Five microliters of the resulting solution was used as target DNA for a subsequent PCR amplification. In all cases, at least two bands showing the same electrophoretic migration were extracted from different lanes and amplified independently, and 100% identity was confirmed. The purity and correct migration within the gels of each fragment were verified by a new DGGE. Reamplified PCR products showing single bands during DGGE analysis were purified as described above and sequenced with the 518r primer. Assignment of 16S rRNA gene sequences from DNA eluted from selected excised DGGE bands was performed using the Ribosomal Database Project (RDP) classifier (http://rdp.cme.msu.edu/) with the confidence threshold set above the default value of 80% (Wang *et al.*, 2007).

Data analysis

All statistical analyses were performed using PAST, PALAEONTOLOGICAL STATISTIC software, version 1.93 (http://folk.uio.no/ohammer/past). Shifts in the composition between samples from the same reactor or between samples from the four reactors were visualized using nonmetric multidimensional scaling (NMDS), based on a similarity matrix computed using the Bray–Curtis measure for q-PCR data. As a measure of the goodness of fit of the reproduced distances to the observed distances, the stress value was used. Stress values < 0.2 indicate that the NMDS plot is an acceptable representation of the original data. Hierarchical cluster generated using group-average linking based on the Bray–Curtis similarity matrix was superimposed on the NMDS plot to form ellipses at arbitrary cut-off values of the dendrogram (Thakuria *et al.*, 2009).

A nonparametric analysis of similarity (ANOSIM) was used to test the null hypothesis that the different phenol loading had no effect on the abundance distribution of the LmPH gene within reactors. A similarity of percent analysis (SIMPER) was used to assess which LmPH genotypes were primarily responsible for the differences detected between groups of samples (Clarke, 1993).

The positions and intensities of bands on the DGGE were determined with the aid of GENE TOOLS software (Syngene). Nonmetric multidimensional scaling was performed using Bray–Curtis distance measures.

Isolation of phenol-degrading bacteria

Phenol-degrading bacteria were isolated from the sludge of the lab-scale reactors, except for strains 7ENR100 and LU-E, which were isolated directly from the aeration basin of the activated sludge of the petroleum refinery, which served as the source of the sludge used in the lab-scale experiments. Col8, FC2.13 and MPE1 were isolated in agar MP (minimum phenol) medium (Watanabe *et al.*, 1998) supplemented with 200 mg L^{-1} of phenol; 7ENR100 was isolated in MP agar plus 100 μM of phenol after 10 days of enrichment in liquid medium of the same composition; LU-E was isolated in basal medium supplemented with trace element solution (Zhang *et al.*, 2007) plus 200 mg L^{-1} of phenol. C10 and AZ113 were isolated in a sterilized supernatant of reactor 1 amended with 5 or 500 mg L^{-1} of phenol. All cultures were incubated at 28 °C. Screening of isolates was performed by restriction analysis of LmPH gene amplicon, using RsaI, MboI and AluI separately.

LmPH genes of selected isolates were amplified using LmPH universal primers. Ribosomal 16S DNA from bacterial isolates was amplified using universal bacterial primers (Suzuki *et al.*, 2000). The taxonomic affiliation of isolates was assigned using the taxonomical hierarchy classifier of the RDP II (Cole *et al.*, 2005).

Determination of phenol-oxygenating activities

Bacterial cells were grown for 3 days on mineral MP medium, supplemented daily with 500 μM of phenol at 28 °C and harvested during the exponential growth phase. The dry weight of the cultures was determined after filtration through a 0.4-μm pore-size membrane filter. The phenol-induced specific oxygen utilization rates were measured at 12 substrate concentrations, from 1 to 2500 μM, using a 600 μL microrespirometer cell equipped with a titanium fiber optic probe (Instech Laboratories Inc.). Phenol-oxygenating activity was calculated for each phenol concentration after subtraction of the endogenous oxygen consumption rate. Data were fitted to the Haldane kinetic model using a nonlinear regression method

$$V = \frac{V_{\max} S}{K_s + S + S^2/K_{si}}$$

where K_s is the half-saturation constant, K_{si} the inhibition constant and $V_{\max}$ the maximum specific activity.

Determination of growth kinetics

Bacterial growth was measured spectrophotometrically in flat-bottomed 96-well microtiter plates, according to Viggor *et al.* (2008). Cells were incubated at 30 °C and 200 r.p.m., in triplicate. Absorbance was measured at 492 nm. The specific growth rates (μ) of cultures grown on different initial phenol concentrations, from 0.2 to 10.6 mM, were calculated from the slope of the exponential growth phase of absorbance growth curves. Data were fitted to the Aiba–Edwards equation

$$\mu = \frac{\mu_{max} S}{K_{sg} + S} \exp\left(\frac{S}{K_i}\right)$$

where μ_{max} is the maximum specific growth rate, S the initial concentration of phenol, K_{sg} is the half-saturation constant and K_i is the inhibition constant.

Nucleotide sequence accession numbers

The sequences of the LmPH gene fragments obtained in this study have been deposited in the GenBank database under accession no. GQ870343–GQ870414. Partial 16S rRNA gene sequences of bacterial isolates have been deposited in the GenBank database under accession no. GQ870415–GQ870421.

Results

Phenol degradation in bioreactors upon a stepwise increase in the phenol loading rate

Phenol degradation rates and the residual concentration of phenol in the supernatants of the two replicate activated sludge reactors subjected to step increases in the feed phenol concentration are shown in Fig. 1a. The phenol biodegradation rate started at initial values of approximately 30 mg phenol g^{-1} dry weight min^{-1}. Correspondingly, phenol levels in the reactor effluents were below the detection limit. After the inlet phenol concentration was increased fivefold to 1.25 g L^{-1}, the phenol degradation rate decreased slightly, but the phenol was still completely degraded. Further inhibition of the phenol degradation rate was observed as the phenol concentration in the feed increased to 2.5 g L^{-1}. At that inlet concentration, the phenol concentration in the effluents increased transiently, before declining back below threshold values. Almost complete inhibition occurred at a phenol concentration of 3.75 g L^{-1} (Fig. 1a); thus, phenol could not be removed completely, and its concentration increased continuously with time. Upon reverting to loading rates of 250 mg L^{-1}, the system regained the ability to degrade phenol and phenol was no longer detected in the effluent.

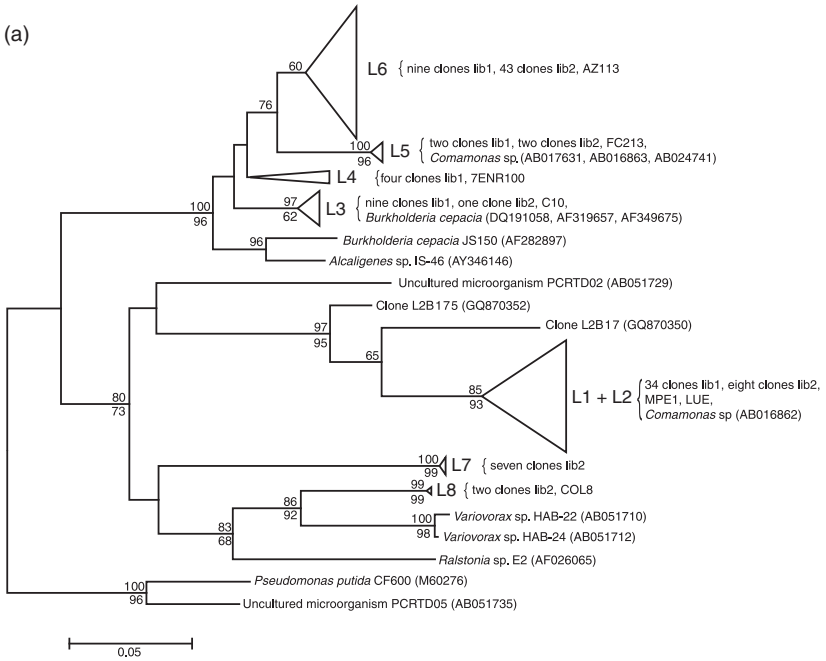
Biomass concentration, estimated as mixed liquor suspended solids, increased initially as the phenol concentration was increased to 1.25 g L^{-1}, remained approximately constant at around 1200 mg L^{-1} at 2.5 g L^{-1} of phenol and declined smoothly at a higher phenol concentration (Fig. 1b).

Phenol hydroxylase gene diversity

The genetic diversity of phenol-degrading bacteria determined in this work extended a previous analysis of a clone library containing a fragment of the gene encoding the largest subunit of the multicomponent phenol hydroxylase (LmPH). That library had been constructed using DNA extracted from activated sludge reactors receiving 250 mg L^{-1} of phenol (Basile & Erijman, 2008). In this work, a new clone library was constructed using DNA extracted from the same reactors at the time that the concentration of phenol in the feed was 2.5 g L^{-1}. LmPH gene sequences from both clone libraries were pooled and divided into subgroups with a genetic distance of 0.11 (Fig. 2). Despite the observed heterogeneity of the nucleic acid sequences, genotypes L1 and L2 clustered together at the protein level, considering a similarity cut-off of 10% of the deduced amino acid sequences.

Based on the predicted amino acid sequences, the LmPHs classified as low K_s-type (Futamata *et al.*, 2001), i.e. phenol-degrading bacteria possessing a high affinity for phenol. Notably, the sequences of the two novel groups found in this study present unique features, which deviate from the consensus amino acid sequence of the low K_s-type bacteria (Fig. 2b). The L8 group has the DP residues in position 252 (numbering of the DmpN sequence; Nordlund *et al.*, 1990), typical of low K_s-type bacteria, but residue 217 is F, a signature amino acid of the high K_s-type bacteria, and residue 117 is M, an attribute that was found only in the LmPH of *Variovorax* strains with distinctive kinetic traits for the degradation of trichloroethylene (Futamata *et al.*, 2005). In the L7 group, the P at position 253 is replaced by an E, characteristic of medium K_s-type and high K_s-type bacteria (Futamata *et al.*, 2001).

Signature sequences in each of the eight LmPH genotypes found in the DNA libraries were targeted with newly designed primers (Table 1) and used for q-PCR analysis. The structures of the phenol-degrading community were similar between identically operated bioreactors at the LmPH gene level at every concentration of phenol in the feed (Fig. 3). Specific primers were also used to quantify medium K_s-type and high K_s-type bacteria. The former were not detected, whereas the latter increased at higher phenol concentrations (Fig. 3). The expression of all LmPH groups was verified by the detection of mRNA by reverse



(b)

115–124	129–150	177–184	214–223	246–258		
GFAHVGRQYP	VACMMQSLDEIRHAQTQIHSLS	SFFDDAVS	GAAYNGDMGA	KFILEQDPDNLPI	L1	
GFAHVGRQFP	VACLMQSLDEIRHAQTQIHSLS	SFFDDAVT	GAAYNGDMGA	KFILEQDPDNLPI	L2	
GFAHVGRHFT	VACQMQSIDELRHFQTEMHALS	SFFEDAAT	GAAYNGDMST	KFMLEQDPDNVPI	L3	
GFAHVGRHFT	VAAQMQSIDELRHCQTETHALS	SFFEDAAT	GAAHNGDMST	KFMLEQDPANVPI	L4	
GFAHAGRHFT	VAAQMQSIDELRHFQTETHALS	SFFEDALS	GAAHNGDLST	KFMLEQDPGNVPI	L5	
GFAHVGRHFT	VAAQMQSIDELRHFQTETHALS	SFFEDAMT	GAAHNGDLST	KFMLEQDPANVPI	L6	
HFAHLARHLP	VAAQMQSIDELRHCQTQIHTIS	SFFDDATS	GAAYNGDMAT	RFLLEQDEANVPI	L7	
GYAMAGRNFR	IASQMQSIDELRHSQTQIHTIS	SYFDDAMS	GAAFNGDMAT	KFLLEQDPDNLPI	L8	
GYAMAGRSLR	VACQMQSIDELRHAQTQFHTIS	SYFEDAMS	GAAYNGDMAT	KFILEQDPDNVPI	*Variovorax* HAB-22	
GFAHAGRHFT	VAAQMQSIDELRHFQTETHALS	SFFEDALS	GAAHNGDLST	KFMLEQDPGNVPI	*Comamomas* R2 (low K_s)	
GFSRVGRQFS	VACQMQAIDELRHVQTQVHAMS	SYMDDART	GAAYNGDMAT	KFMLEQHEDNVPI	*Ps.* CF600 (moderate K_s)	
GFARVGRQFS	IACQMQIADELRHVQTQIHAMS	SFFEDART	GAAFNGDMAT	KFLLEQHEDNVPI	PCRTD05 (high K_s)	
GFAHVGRQFP	VACLMQSLDEIRHAQTQIHSLS	SFFDDALT	GAAYNGDMGA	KFI	MPE1	(L1+L2)
GFAHVGRQFA	VACQMQAVDEIRHFQTQVHSLS	SFFDDAVS	GAAYNGDMGA	KFILEQDPD	LUE	(L1+L2)
GFAHVGRHFT	VACQMQSIDELRHFQTEMHALS	SFFEDAAT	GAAYNGDMST	KFMLEQDP	C10	(L3)
GFAHVGRHFT	VAAQMQCIDELRHCQTETHALS	SFFEDAST	GAAHNGDMST	KFMLEQD	7ENR100	(L4)
GFAHAGRHFT	VAAQMQSIDELRHFQTETHALS	SFFEDALS	GAAHNGDLST	KFMLEQDP	FC213	(L5)
GFAHVGRHFT	VAAQMQSIDELRHFQTETHALS	SFFEDAMT	GAAHNGDLST	KFMLEQ	AZ113	(L6)
GYAMAGRNFR	IACQMQSIDELRHSQTQIHTIS	SYFDDAMS	GAAFNGDMAT	KFLLEQDP	COL8	(L8)

Fig. 2. (a) Unrooted tree based on the translated amino acid sequences of 470 bp fragments of PCR-amplified LmPH genes. Neighbor joining (top) and parsimony (bottom) bootstrap values based on 100 trials are indicated at nodes. The bar is equal to 0.05 substitutions per site. (b) Sequence analysis of amino acid residues found in the deduced sequences of LmPHs determined in this study. Signature sequences for LmPHs with different affinities for phenol are underlined. The regions used for designing the genotype-specific primers are boxed. The numbers above the sequences correspond to the numbering of the DmpN sequence (Nordlund *et al.*, 1990).

transcription-PCR using group-specific primers at every sampling point (data not shown).

Shifts in LmPH genotype abundances were visualized in two-dimensional ordination graphs, using NMDS. Data from the two reactors were close to each other at all phenol concentrations, indicating that the LmPH populations shifted similarly in both replicates (Fig. 4). LmPH-containing populations from the control reactors were more widely spread across the NMDS plot, together with the bacterial communities of the two treated reactors at the time that they also received a phenol concentration of 250 mg L^{-1} in the feed. The ANOSIM confirmed that the distributions of LmPH abundances from the replicate-treated reactors were more similar to each other than to the same reactor at different phenol loadings (the global R statistic for the data was 0.74, $P = 0.0001$). SIMPER revealed that three genotypes (L1, L5 and L8) contributed most to the shift in the composition between reactors exposed to different phenol loadings (Table 2).

Community structure studied by 16S rRNA gene analysis

DGGE analysis of PCR-amplified partial 16S rRNA genes from the two treated and two control reactors was conducted to reveal changes in the overall bacterial community structure (Fig. 5). The NMDS analysis of DGGE banding patterns was qualitatively similar to the LmPH gene-based NMDS plot, in that the samples were close together according to the concentration of phenol in the feed (Fig. 6). However, clustering of the total bacterial community patterns in replicate reactors was less marked than that seen in LmPH genotypes. This is likely due to the overlapping of stochastic variations, reflected by the high dispersion of the

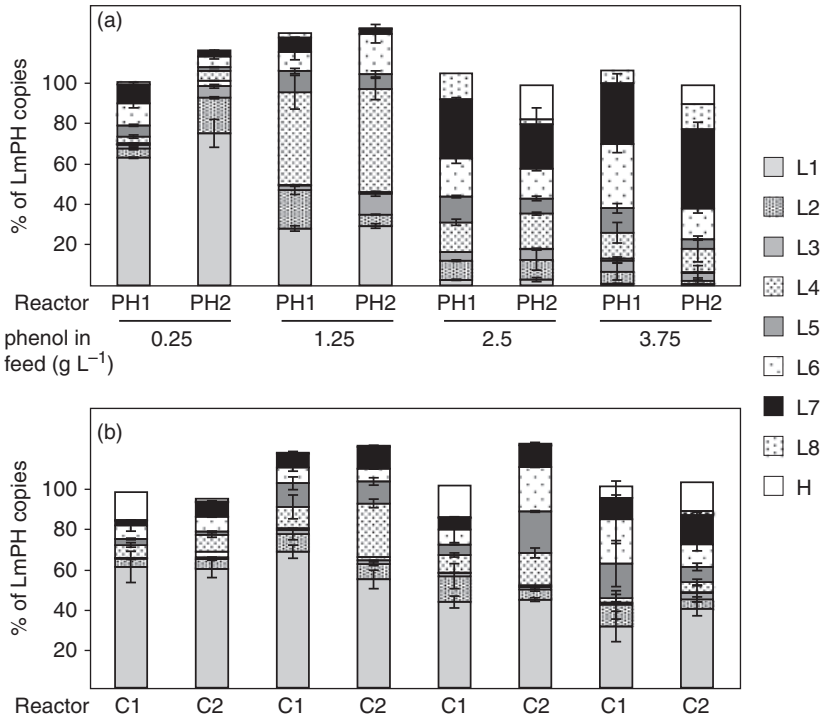


Fig. 3. Relative abundance of LmPH genotype copy numbers in treated activated sludge reactors, at the different initial phenol concentrations (a) and in samples taken in parallel times from control reactors (b). Data are expressed as a percentage of the total LmPH copies, determined using a mixture of primers LmPH Uf 1, 2 and 3, and LmPH Ur. H corresponds to the high K_s-type bacteria. C1 and C2, control reactors; PH1 and PH2, treated reactors. Error bars indicate SD.

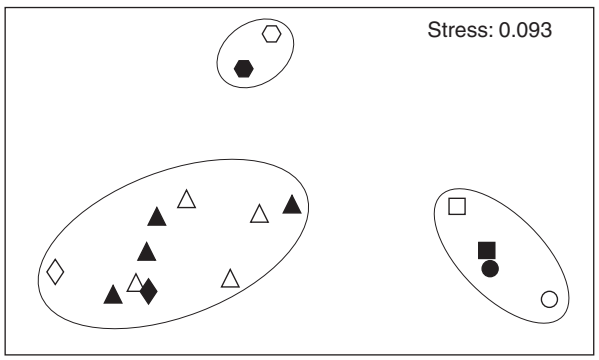


Fig. 4. Representation of the q-PCR data of Fig. 3 by a two-axis nonmetric multidimensional scaling based on a matrix of Bray–Curtis similarities. Samples from replicate reactors are indicated by solid symbols and open symbols. Triangles correspond to control reactors. Diamonds, hexagons, squares and circles correspond to treated reactors at the initial phenol concentrations of 0.25, 1.25, 2.5 and 3.75 g L^{-1}, respectively. Ellipses represent superimposed hierarchical clusters (at a similarity level of 60%), deduced using group-average linking based on the same Bray–Curtis similarity matrix.

control communities subjected to the same operational conditions throughout the experiment.

Several bands that displayed changes as a function of phenol amendment were excised from the DGGE for sequence analysis (Fig. 5). Comparison with the 16S rRNA genes in the GenBank and RDP II databases indicated that the majority of bands that appeared at a phenol concentration of 1.25 mg L^{-1} and persisted at all phenol concentrations belonged to the *Betaproteobacteria*, particularly *Comamonadaceae*, including the genus *Comamonas* and *Delftia* (Table 3). The remaining band was related to the order *Actinomycetales*. The sequence of a band that appeared at a low phenol concentration, but was no longer detectable at the highest phenol concentration belonged to *Bacteroidetes*. A band of low intensity detected only in reactors exposed to 2.5 g L^{-1} of phenol was affiliated to *Sphingobacterium* (*Bacteroidetes*). Two bands, which appeared only in reactors subjected to 3.75 mg L^{-1} of phenol, were related to the family *Alcaligenaceae* and the order *Rhizobiaceae*. Bands a, b and c were always detected in controls, but disappeared at different phenol concentrations, suggesting inhibition by phenol.

Specific growth rates and oxygen consumption kinetics of representative isolates

We hypothesized that the dominance of particular bacteria at the different loadings of phenol could be attributed to their functional traits related to the affinity and/or the tolerance to phenol concentration. In order to test this hypothesis, we isolated representatives from each group of the LmPH gene, and evaluated their kinetics and growth behavior with phenol as the source of carbon and energy.

Table 4 presents the taxonomic affiliation, the specific growth rates and the oxygen utilization kinetics of seven

Table 2. ANOSIM and SIMPER global and pairwise comparisons of LmPH gene distribution under different phenol loadings

	ANOSIM		SIMPER: percentage contribution of LmPH genotypes to dissimilarities between communities under different phenol loadings								
Comparison*	R	*P*	L1	L2	L3	L4	L5	L6	L7	L8	H
Global	0.74	0.0001	**37.1**	6.0	4.1	1.1	**16.9**	5.9	8.2	**15.5**	5.3
0.25 vs. 1.25	0.88	0.021	**22.1**	7.9	7.8	1.1	**41.7**	6.0	7.3	5.2	0.9
1.25 vs. 2.5	0.98	0.023	**46.5**	3.4	3.4	1.1	8.7	5.9	7.4	**16.9**	6.6
2.5 vs. 3.75	0.99	0.023	**41.7**	3.7	2.8	0.9	5.6	5.3	9.5	**22.5**	8.1

*Only the pairwise comparisons that were significantly different are shown. Bold values indicate the genotypes that contribute most to the observed differences.

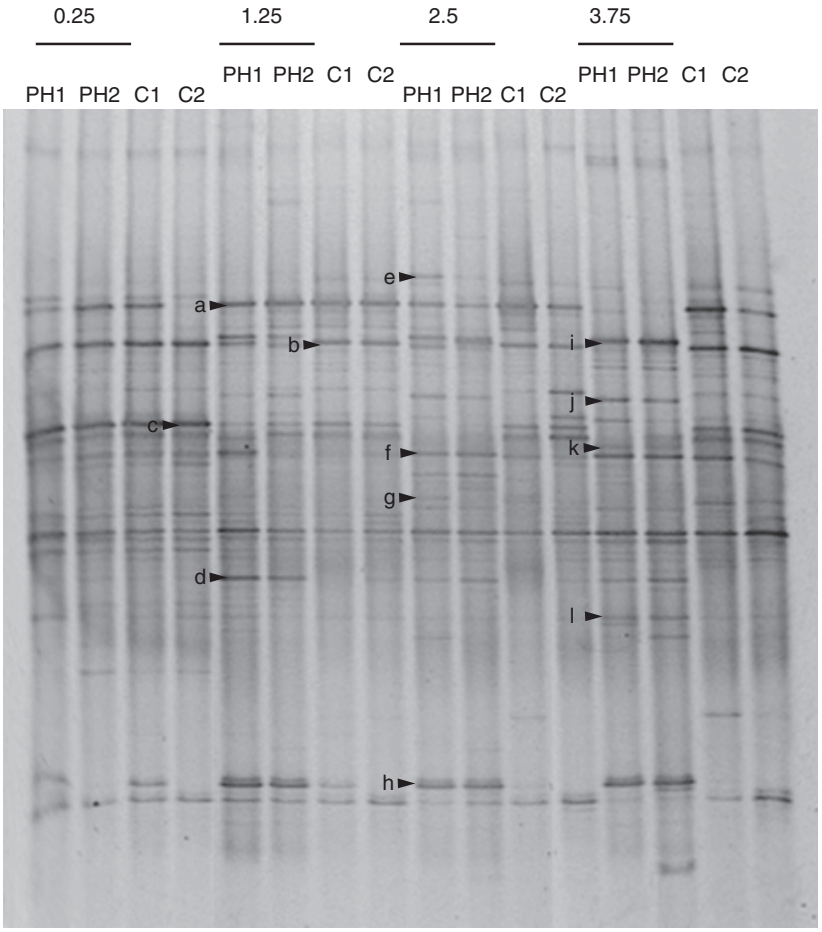


Fig. 5. Negative images of SYBR green-stained DGGE gels showing the band pattern of the 16S rRNA gene. The numbers above indicate the concentration of phenol in the feed. Arrows indicate the bands that were sequenced.

phenol-degrading isolates, containing different LmPH genotypes. Similar to the case of the bands retrieved from the DGGE gels, the isolated strains were affiliated mostly with the *Betaproteobacteria*, except for L4, identified as *Acinetobacter* sp., a member of the *Gammaproteobacteria*.

The V3 regions of all the isolates were amplified and run in a DGGE, together with the samples of the phenol-amended reactors (data not shown). Isolates L5 and L6 showed mobilities similar to the DGGE bands d and f, respectively, and had identical 16S rRNA gene fragment sequences (Fig. 5 and Table 3), confirming that they were important members of the reactors subjected to phenol amendment.

The K_s values for the phenol-degrading activity were within a relatively small range: from 2.3 to 6.6 μM. The inhibition constant diverged over one order of magnitude:

from 700 to 6700 μM. With the exception of L1, which had a higher affinity for growth on phenol, all the strains had an approximately similar K_{sg} in the range of 1.3–2.1 mM.

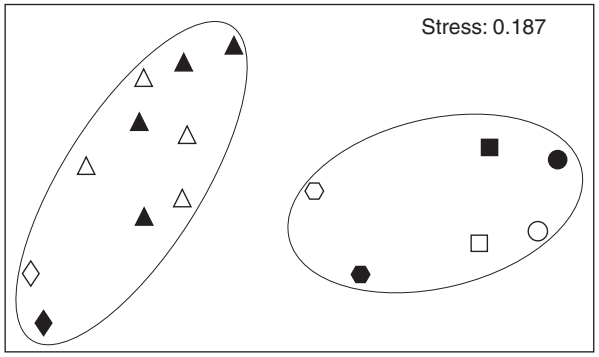


Fig. 6. NMDS diagrams relating the bacterial 16S rRNA gene on the basis of the DGGE gels shown in Fig. 5, using a matrix of Bray–Curtis dissimilarities. Symbols and ellipses were drawn using the same criteria as in Fig. 4.

Discussion

It has been proposed that one of the engineering objectives in biological wastewater treatment should be to maximize diversity. Hence, higher species richness would improve process stability through functional redundancy (Briones & Raskin, 2003; Franklin & Mills, 2006; McMahon *et al.*, 2007). More generally, the generation and maintenance of genetic variation appears to be a widespread ecological strategy of bacterial populations, which may help to sustain stability in situations of uncertain environmental change (Heuer *et al.*, 2008). Importantly, stability usually results from functional redundancy and functional niche complementation rather than population diversity *per se*. However, most of the diversity–stability studies performed to date have focused on the ribotype diversity, from which functional redundancy could only be deduced. This work, which addressed diversity directly at the functional level, indicates that lab-scale reactors may support many ecological niches,

Table 3. Summary of DGGE band analysis

Bands	Taxon affiliation (RDP II classifier)*	Most closely related bacterial sequence†	GenBank accession no.	Identity
a	*Sphingobacteriales*	Uncult. *Bacteroidetes*	FM253021	98% (125/127)
b	*Bacteroidetes*	Uncult. *Bacteroidetes*	FJ517722	100% (133/133)
c	*Simplicispira*	Uncult. betaproteobacterium	GU123159	98% (143/145)
d	*Comamonas*	*Comamonas testosteroni* GAD4	FJ639332	100% (133/133)
e	*Sphingobacterium*	*Sphingobacterium* sp. GF2B	FJ548749	100% (127/127)
f	*Delftia*	*Delftia* sp. LP2MM	GU272362	100% (139/139)
g	*Bacteroidetes*	Uncult. *Bacteroidetes*	EF179856	99% (110/111)
h	*Corynebacterineae*	*Rhodococcus pyridinivorans* SB 3094	GU191923	100% (129/129)
i	*Comamonadaceae*	Uncult. betaproteobacterium	FM252654	100% (142/142)
j	*Comamonas*	*Comamonas* sp. PG-0	AY566581	100% (123/123)
k	*Alcaligenaceae*	*Alcaligenes* sp. C4	AF384197	97% (115/118)
l	*Rhizobiaceae*	*Ochrobactrum* sp. ITRH1	FJ013273	97% (118/121)

*Using the program 'classifier' at the RDP II website (http://rdp.cme.msu.edu/classifier/classifier.jsp) and a minimum default confidence threshold of 80%.

†Using BLASTN against the NR database at NCBI.

Table 4. Characteristics of the isolated strains

			Phenol-oxygenating activity (Haldane equation)			Growth on phenol (Aiba–Edwards equation)		
Isolate names	LmPH genotypes	Affiliations*	K_s (μM)	K_{si} (μM)	V_{max}†	K_{sg} (mM)	K_i (mM)	μ_{max} (h^{-1})
MPE-1	L1	*Alicycliphilus*	4.1 ± 0.8	2200 ± 600	340 ± 20	0.3 ± 0.2	8.0 ± 2.7	0.74 ± 0.19
LU-E	L2	*Brachymonas*	2.3 ± 0.5	3000 ± 900	103 ± 5	2.1 ± 1.7	3.6 ± 0.9	1.10 ± 0.64
C-10	L3	*Pigmentiphaga*	5.3 ± 1.8	3300 ± 1500	420 ± 30	ND	ND	ND
7-ENR100	L4	*Acinetobacter*	2.6 ± 0.9	1100 ± 400	250 ± 20	1.5 ± 0.6	5.1 ± 0.8	0.34 ± 0.09
FC2-13	L5	*Comamonas*	6.6 ± 1.8	700 ± 200	500 ± 40	1.3 ± 0.9	7.9 ± 2.5	0.40 ± 0.15
AZ1-13	L6	*Delftia*	6.5 ± 1.3	1300 ± 300	330 ± 20	1.7 ± 0.9	3.4 ± 0.4	0.35 ± 0.28
COL-8	L8	*Pigmentiphaga*	5.3 ± 1.0	6700 ± 2500	260 ± 10	1.7 ± 0.8	6.7 ± 1.4	0.42 ± 0.13

*Affiliation was assigned using the classifier of the RDP (http://rdp.cme.msu.edu/b), based on 700-bp sequences with a confidence threshold of 95% for all isolates, except for MPE-1, which was classified with a confidence of 90%.

†V_{max}, μmol oxygen consumed min^{-1} g^{-1} dry weight.

ND, not determined.

allowing the maintenance of a high diversity of ecotypes through varying concentrations of phenol.

The proportions of some phenol hydroxylase genotypes peaked at an intermediate phenol concentration, whereas others decreased with increasing phenol concentration. The reproducible quantitative changes in the concentration of the genes encoding phenol hydroxylase accompany changes in the phenol degradation rates, indicating a community structure–function relationship. The reproducibility of bacterial populations in replicate reactors has been recognized as an indication of the deterministic selection of organisms according to their fitness to fill empty niches (Curtis & Sloan, 2004; Lozada *et al*., 2006; McGuinness *et al*., 2006; Ayarza *et al*., 2010; Falk *et al*., 2009; Wittebolle *et al*., 2009). The similarity in the patterns of LmPH abundance was also maintained at concentrations at which the sludge was exposed to sufficiently high concentrations of phenol that may approach partial cell leakage and cell lysis, leading ultimately to process breakdown (Watanabe *et al*., 1996, 1999), indicating that tolerance to phenol toxicity could be partially driving the population shifts.

At the same time, a drift due to stochastic forces was also inferred from the divergence in the trajectories of both LmPH gene-based analysis (Fig. 4) and 16S rRNA gene-based analysis (Fig. 6) in the replicated communities of the control reactors. Because the generation of variation is a stochastic process driven by random events such as immigration and extinction, different communities with similar fitness may develop in identical physical environments (Woodcock *et al*., 2007). The role of neutral processes in shaping the bacterial community structure has been deduced from the divergence in the composition of microbial communities in replicated systems in functionally stable anaerobic reactors (Fernandez *et al*., 1999), activated sludge (Kaewpipat & Grady, 2002), denitrifying bioreactors (Gentile *et al*., 2007) and replicate microcosms provided with discrete pulses of protein (Carrero-Colon *et al*., 2006; Konopka *et al*., 2007). A stochastic model of community assembly explained the coexistence of species in a homogeneous environment, i.e. without patchiness or spatial or temporal environmental heterogeneity (Sloan *et al*., 2006).

Amidst the debate over the relative importance of both neutral and niche-oriented dynamics over community composition, it has been suggested that the neutral model is a special case, in which stabilizing mechanisms are absent, and species have equivalent fitness (Adler *et al*., 2007). Chase hypothesized that the influence of each factor depends on the harshness of the physical habitat: stochastic processes prevail in benign habitats, where several functional redundant generalists may occupy available niches, whereas deterministic processes prevail under harsh environmental conditions, where specialized niches select for species carrying suitable traits (Chase, 2007). This prediction was confirmed by van der Gast *et al*. (2008), who examined the effects of temporal turnover on bioreactors under increasing selective pressure exerted by a gradient of increasing industrial wastewater concentrations and decreasing municipal wastewater.

The implication is that the requirement of specialized populations to degrade and/or to tolerate phenol may explain why phenol-degrading communities are more reproducible, compared with the larger divergence observed in the fingerprinting pattern of the total bacterial community (Fig. 6) and also in nonselective environments.

We have observed that bacterial populations dedicated to a specific function manage to coexist under varying environmental conditions in a relatively homogeneous environment. From a practical point of view, the idea that species that increase their abundance in response to a higher phenol concentration can fill the functional niches occupied by the more sensitive species has been discussed in terms of the effectiveness of bioaugmentation strategies with a diverse community of phenol degraders that could perform under variable field conditions (Jiang *et al*., 2007).

In an attempt to elucidate how the various genotypes partition as a function of the feed phenol concentration, we isolated phenol-degrading bacteria containing seven out of the eight LmPH genotypes detected in our LmPH clone libraries.

Futamata *et al*. (2001) showed that the phylogenetic classification of different types of phenol hydroxylase genes correlated to the kinetic properties of phenol-degrading bacteria. In the current study, we have detected the presence of two novel populations of LmPH genotypes, for which the deduced amino acid sequences include mixed features of different phenol hydroxylase gene types. Yet the growth rate and kinetic parameters of the phenol-degrading activity determined for our isolates did not correlate with the relative abundance of the corresponding LmPH genotypes at the different phenol loadings. Thus, in agreement with observations made previously by others (Zhang *et al*., 2004; Futamata *et al*., 2005; Viggor *et al*., 2008), we propose that the relationship between the LmPH gene type and the kinetic properties of the bacteria is less certain than has been maintained hitherto, at least in their traits related to the degradation of phenol.

Most of the isolates possessing LmPHs with a low K_s, as well as the majority of DGGE bands differentially observed in phenol-exposed reactors, belonged to the *Betaproteobacteria*. This result is in agreement with the fact that phenol-degrading bacteria with low-K_s-type LmPH are members of the *Betaproteobacteria*, such as *Comamonas testosteroni* R5 and *Burkholderia cepacia* G4 (Futamata *et al*., 2001). The dominance of other members of the *Proteobacteria* within the family *Alcaligenaceae* and the order *Rhizobiaceae*, encouraged by the high phenol concentration,

may be rationalized on the basis of phylotypes that are either adapted or resistant to a high phenol concentration. However, the presence of a dominant band should not be taken as conclusive evidence of the ability of the phylotype to degrade phenol. Futamata *et al.* (2001) have observed a correspondence between the bacterial isolation efficiency and the intensity of DGGE bacterial populations in a chemostat enrichment. We have not detected such a tendency, probably due to the fact that in this work phenol was not the only source of carbon and energy supplied to the reactors.

The phenol-degrading bacteria isolated in the current study exhibited a rather narrow range of physiological diversity. This contrasts with the finding of Jiang *et al.* (2006), who isolated 10 bacterial strains from phenol-degrading sludge granules with a wide range of physiological diversity. Because co-occurrence may be explained by niche differentiation in competitive environments (Chesson, 2000; Shea *et al.*, 2004; Haddad *et al.*, 2008), the difference could be attribute to the higher niche heterogeneity in the granules compared with the activated sludge (Jiang *et al.*, 2006; Liu *et al.*, 2009).

It has to be noted that only two of the isolated strains were detected in the DGGE fingerprinting analysis of the reactors. Our working hypothesis was that the distinct groups with a genetic distance of 11% most likely represented ecologically relevant variants of the enzyme. Nevertheless, the phylogenetic tree (Fig. 3) shows that the LmPH diversity within each genotype was very high; therefore, we could not make certain the actual abundance of each strain in the reactors. Because it cannot be assumed that distinct populations carrying the same genotype were ecologically interchangeable, and thus members of the same population, the true representativity of the isolated strains belonging to the other five groups could only be inferred. As is the case for many other metabolic pathways, the ability to degrade phenol may have been acquired by bacteria belonging to different taxa through events of horizontal transfer (Koonin *et al.*, 2001). A lack of correlation between the phylogeny of phenol-degrading isolates of *Alcaligenes* sp. and their LmPH genotypes was attributed to horizontal transfer (Zhang *et al.*, 2004). In *Alcaligenes faecalis*, an ORF encoding a transposase of insertion sequences (IS) 4 family was found downstream of the phenol hydroxylase gene cluster containing the LmPH gene (Zhu *et al.*, 2008), suggesting that the whole phenol catabolic gene cluster may have been transferred between different bacteria. Lateral transfer of the LmPH gene between bacterial strains would allow ecologically distinct populations to occupy similar ecological niches, leading to the coexistence of a high diversity of species capable of performing similar tasks.

Yet the coexistence of ecologically similar or equivalent species, which do not display differences required by theory, has long been recognized (Pielou, 1978; Clark *et al.*, 2007). The analysis of the biochemical properties performed on the isolates carried out in the present study supports the idea that the reliance on the phenol-degrading properties of the isolates obtained does not allow the assessment of the ability of a particular strain to become a dominant member of the community under the different environmental conditions. Recently, the high dimensionality hypothesis (Clark *et al.*, 2007) has been put forward to explain the coexistence of closely related species in a community, by postulating 'hidden' additional niche dimensions, which may sustain the unobserved differences. Thus, further attempts to obtain pure cultures for physiological analysis of ecologically relevant strains, in conjunction with novel genomics tools, might help in determining whether the rules governing bacterial assembly could be elucidated through additional traits.

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