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Parvibaculum lavamentivorans converts linear alkylbenzenesulphonate surfactant to sulphophenylcarboxylates, α,β-unsaturated sulphophenylcarboxylates and sulphophenyldicarboxylates, which are degraded in communities

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ABSTRACT

Aims: The aims were to test whether *Parvibaculum lavamentivorans*^{T} degraded commercial linear alkylbenzene-sulphonate (LAS) surfactant via ω-oxygenation and β-oxidation to sulphophenylcarboxylates (SPCs), whether the organism was widespread and reisolable, and whether the degradative community used the 4-sulphocatechol 1,2-dioxygenase to cleave the aromatic ring from LAS.

Methods and Results: Heterotrophic *P. lavamentivorans*^{T} converted LAS (side chain length C_{10}–C_{13}) to SPCs (C_4–C_{13}), α,β-unsaturated SPCs (C_4–C_{13}) and sulphophenyldicarboxylates (SPdCs) (at least C_8–C_{12}). Identifications came from high performance liquid chromatography (HPLC) separation, an electrospray interface and mass spectrometry. No evidence for other paths was found. The degradation of LAS in trickling filters inoculated with environmental samples always showed transient SPC intermediates (HPLC) and the presence of the *P. lavamentivorans* morphotype in the community. One new isolate was obtained. A community able to mineralize LAS contained 4-sulphocatechol-1,2-dioxygenase at high specific activity.

Conclusions: *Parvibaculum lavamentivorans*^{T} degrades commercial LAS via ω-oxygenation, oxidation and chain shortening through β-oxidation to yield a wide range of SPCs. The latter are degraded in bacterial communities which contain organisms like *P. lavamentivorans*, and which utilize sulphocatechol dioxygenase for ring cleavage.

Significance and Impact of the Study: There is one widespread pathway to degrade LAS. Any traces of LAS and larger amounts of SPCs in the effluent from sewage works are exposed to degradative organisms in acclimated and pristine environments. These degradative reactions can now be studied in pure cultures.

Keywords: α,β-unsaturated sulphophenylcarboxylates, 2-dioxygenase, 4-sulphocatechol 1, linear alkylbenzene-sulphonate surfactant, microbial communities, *Parvibaculum lavamentivorans*.

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INTRODUCTION

Some 2·5 million tonnes of commercial linear alkylbenzenesulphonate (LAS) surfactants (the annual production; Schulze 1996) are degraded every year, but our understanding of the degradative process is largely limited to saying that a microbial community is essential (Jiménez *et al.* 1991; Sigoillot and Nguyen 1992; Hrsák and Begonja 1998, 2000; Schleheck *et al.* 2000; Schulz *et al.* 2000). Little is known about the degradative pathway(s) involved (Cook 1998; Eichhorn and Knepper 2002), which are usually simplified to the scheme of ω-oxygenation (and oxidation) to long-chain sulphophenylcarboxylates (lc-SPC), β-oxidation via medium-chain sulphophenylcarboxylates (mc-SPC) to short-chain sulphophenylcarboxylates (sc-SPC) followed by the complex of ring cleavage and desulphonation (Swisher 1987; Schöberl 1989; Cook 1998); an extension of this concept involves two ω-oxygenations and sulphophenyldicarboxylate (SPdC) intermediates, which are also observed (di Corcia *et al.* 1994, 1999). This hypothesis was markedly strengthened with the discovery of α,β-unsaturated SPCs (Eichhorn and Knepper 2002) in mixed cultures. One of the alternative degradative pathways involves initial desulphonation of LAS (Campos-García *et al.* 1999). The degradative pathway for the sc-SPC has been subject to much speculation (Cook 1998).

A methanotroph, which oxygenates and oxidizes 2-(4-sulphophenyl)dodecane (2-C12-LAS) to the corresponding lc-SPC [11-(4-sulphophenyl)dodecanoate] 11-C12-SPC in the presence of methane (strain A in Fig. 1) has been isolated from a LAS-degrading community (Hrsák and Grbic-Galic 1995; Hrsák and Begonja 1998). The lc-SPC is oxidized by (isolated) heterotrophic bacteria in the community to 3-(4-sulphophenyl)butyrate (3-C4-SPC), a sc-SPC, apparently by several spirals of β-oxidation (strain B in Fig. 1). Two tiers of organisms are thus required to generate the sc-SPCs, which are largely degraded in Hrsák's original culture (Hrsák and Grbic-Galic 1995). So a three-tier degradative community (i.e. with strain C in Fig. 1) has to be proposed to achieve the mineralization of LAS when initiated by a methanotroph. Another group of bacteria, the autotrophic ammonia oxidizers, especially *Nitrosospira* sp., causes some disappearance of LAS in culture (Brandt *et al.* 2001). A heterotrophic bacterium, *Parvibaculum lavamentivorans*^{T} utilizes 3-C12-LAS as the sole carbon and energy source, and excretes largely 4-C6-SPC (Schleheck *et al.* 2000, 2003; D. Schleheck, B. Tindall, R. Rosselló-Mora and A.M. Cook, unpublished data) (strain D in Fig. 1); a second tier of organism(s) is obviously required for further degradation of this sc-SPC, which is now known to be fully degraded in a pure culture (strain E in Fig. 1) (D. Schleheck, unpublished data). The sc-SPC whose degradation has been described, the enantiomeric 2-C4-SPC, is catabolized via 4-sulphocatechol and *ortho* ring cleavage by *Delftia acidovorans* SPB1 (strain C in Fig. 1) (Schulz *et al.* 2000). So the earlier speculation (Cook 1998) can be tentatively replaced by some characterized reactions in an organism that represents the final tier in LAS degradation (strain C in Fig. 1).

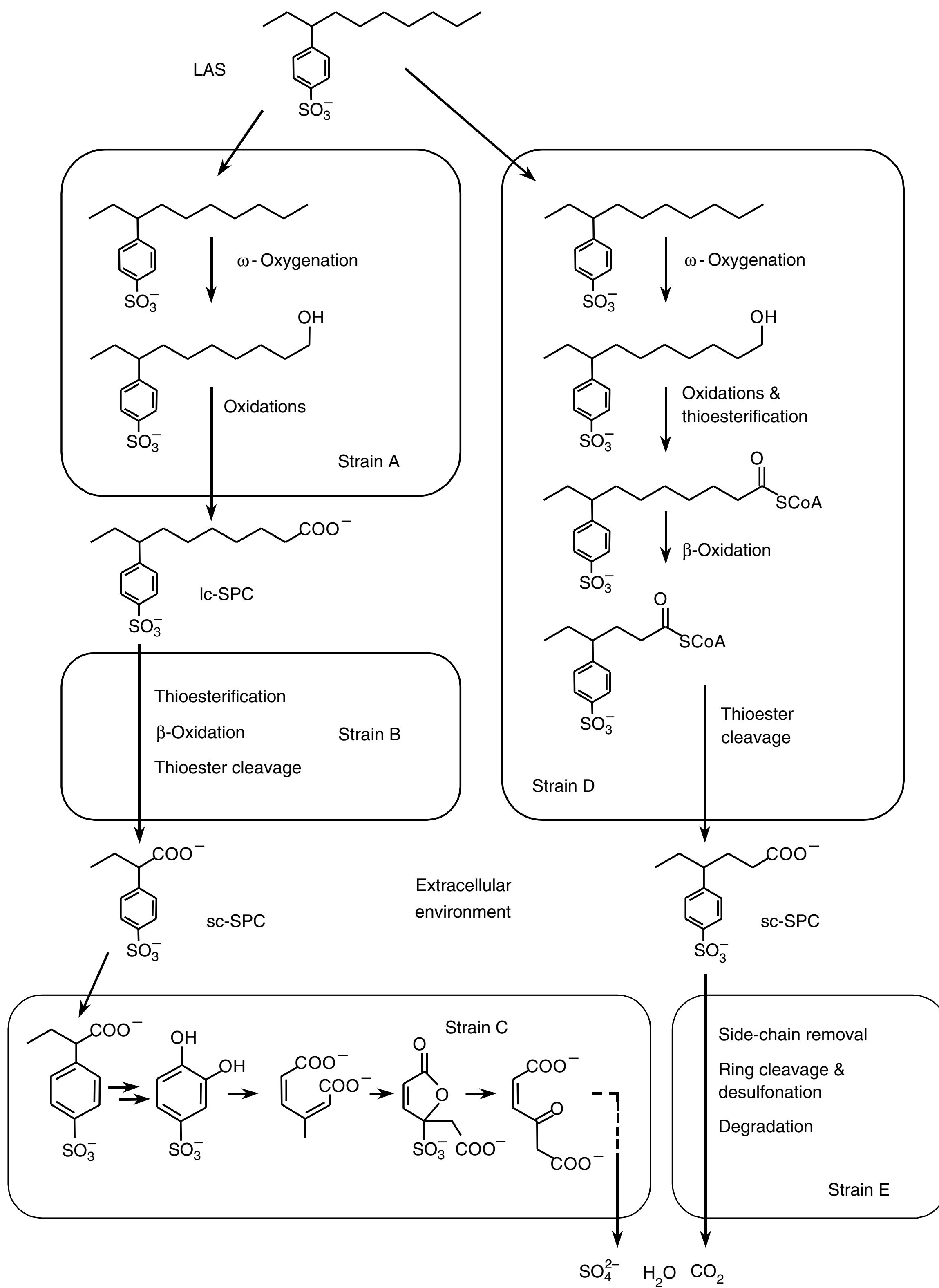
The existence of two communities that degrade individual LAS congeners in two or three tiers does not mean that the updated hypothesis (ω-oxygenation, β-oxidation, *ortho* ring cleavage and desulphonation) is generally correct. Commercial LAS is still quite demanding to analyse by high performance liquid chromatography (HPLC), especially when the SPCs are included (Kölbener *et al.* 1995b), and analysis of the degradation of LAS at this level of resolution is still a rarity (Kölbener *et al.* 1995b; di Corcia *et al.* 1999; Knepper and Kruse 2000; Eichhorn and Knepper 2002). So other pathways are not excluded, although the evidence for ω-oxygenation and β-oxidation in mixed culture has now been markedly improved by the development of an improved HPLC-separation method for LAS, SPCs and SPdCs, and coupled by an electrospray interface to a mass spectrometer (HPLC-ES-MS) (Eichhorn and Knepper 2002).

Evidence is now presented that *P. lavamentivorans*^{T} indeed catalyses ω-oxygenation and β-oxidation, is widespread, and that the scheme of degradation of commercial LAS sketched in Fig. 1, especially the purely heterotrophic arm, is the only significant pathway in samples from pristine and contaminated environments.

MATERIALS AND METHODS

Materials

Experiments were routinely carried out with a typical commercial LAS (C_{10}–C_{13} sidechain length, Marlon A350; Hüls, Marl, Germany) at 100 mg dissolved organic carbon l^{-1} in the mineral salts solution recommended by the Organisation for Economic Cooperation and Development (OECD) for the '300' series (Kölbener *et al.* 1995b). 3-(4-Sulphophenyl)dodecane (3-C12-LAS; >95%) was made available by CONDEA-Vista (Houston, TX, USA); it contained traces of 3-C10-LAS, 3-C11-LAS and 5-C11-LAS as impurities, estimated from their retention times compared with commercial LAS separated by HPLC with a C-18 column (cf. Kölbener *et al.* 1995b). An LAS analogue, linear alkyldiphenyletherdisulphonate (Schleheck *et al.* 2000), was used in some enrichment cultures. It is commercially available as Dowfax 8390 (Dow Chemical, Midland, MI, USA). A sterile, standard mixture of SPCs, SPdCs and α,β-unsaturated SPCs [termed SP(d)Cs] (Fig. 4) was generated by growing *P.*


LAS
SO_3^-
ω- Oxygenation
OH
Oxidations
Strain A
COO^-
lc-SPC
Thioesterification
β-Oxidation
Thioester cleavage
Strain B
Oxidations &
thioesterification
O
SCoA
β-Oxidation
Thioester
cleavage
Strain D
sc-SPC
Extracellular
environment
Strain C
OH
O
Side-chain removal
Ring cleavage &
desulfonation
Degradation
Strain E
SO_4^{2-}
H_2O
CO_2

lavamentivorans[T] in 2 mM LAS salts glass fibre medium, removing cells and glass fibre by centrifugation (10 000 *g*, 4°C, 10 min), and passing the supernatant fluid through a sterile membrane filter of 0·2 μm pore diameter. The three pristine inocula were from Lake Konstanz (profundal sediment from 80 m depth, and littoral sediment) and a forest soil near the University of Konstanz. The agricultural soil was from a field of maize near Konstanz. Sewage sludge was from the waste treatment plants in Moos (rural) and Radolfzell (largely communal), both in south Germany.

Trickling filters

Baumann's laboratory trickling filter, which represents a simplified sewage treatment plant (Kölbener *et al.* 1995b), was used in its simplest form (Schleheck *et al.* 2000); vertically mounted glass tubes containing a polyester fleece on which bacteria immobilized spontaneously. Each inoculum was suspended in the OECD salt solution, to which the fleece was added, and then hung from a hook in the upper stopper on the glass tube. The sterile solution of LAS and filtered air were pumped through needles in the upper stopper, and taken off with a siphon and a needle, respectively, in the lower stopper. The battery of 12 filters was operated at room temperature, *ca* 25°C.

Fig. 1 Presumed major pathway(s) for the conversion of linear alkylbenzenesulphonates (LAS) to short-chain sulphophenylcarboxylates (sc-SPC) and complete degradation, with examples using 2-(4-sulphophenyl)butyrate (2-C4-SPC) and 4-C6-SPC as the sc-SPC. This figure, especially the right-hand side, represents a specific example of the general hypothesis under test. The sketch shows putative communities comprising known bacteria. Strain A is a methanotroph (Hrsák and Begonja 1998), strain B is a heterotroph (Hrsák and Begonja 1998) and strain C is *Delftia acidovorans* SPB1 (Schulz *et al.* 2000), as is strain E (D. Schleheck, unpublished data); strain D is *Parvibaculum lavamentivorans* (D. Schleheck, B. Tindall, R. Rosselló-Mora and A.M. Cook, unpublished data). Commercial LAS in Europe is, ideally, a mixture of four linear alkanes (C_{10}–C_{13}) subterminally substituted with a single 4-sulphophenyl moiety: this means a mixture of 20 compounds, 18 of which are racemic mixtures. There seem to be many SPCs besides 2-C4-SPC, which are subject to degradation by specific cultures (D. Schleheck, unpublished data), so we anticipate that many further organisms are required to degrade LAS fully. For simplicity, no attempt was made to incorporate the degradation of SPdCs into this diagram. A spiral of β-oxidation of the 8-C10-SPC-CoA (shown in strain D) would, by analogy to known systems (Lehninger *et al.* 1993), involve a FAD-linked dehydrogenase to yield the α,β-unsaturated SPC CoA ester, which would be subject to hydratase activity to yield the β-hydroxy CoA ester. This ester would be oxidized by a NAD-dependent dehydrogenase to the β-oxo CoA ester, which would be subject to CoA-dependent thiolysis to yield acetyl-CoA and 6-C8-SPC-CoA. The pathway for the degradation of SPB is from published work (Feigel and Knackmuss 1993; Schulz *et al.* 2000)

Analytical methods

Commercial LAS, SPC and SPdC as well as linear alkyldiphenyletherdisulphonate and its degradative products were routinely separated and determined by gradient elutions (perchlorate/acetonitrile) in reversed phase (C-18) HPLC with diode array detection (Schleheck *et al.* 2000). The method is essentially that of Kölbener *et al.* (1995b), who described that the centrally substituted LAS molecules in a set of isomers eluted before those with peripheral substituents. 4-Sulphocatechol and 3-sulphomuconate in enzyme assays were determined after separation on a β-pm-Nucleodex HPLC column (Schulz *et al.* 2000). Ion chromatography with suppression is described elsewhere (Laue *et al.* 1996). HPLC-ES-MS, with gradient separation (water/acetonitrile modified with triethylamine/acetic acid) on a C-18 column, is described elsewhere (Eichhorn and Knepper 2002).

Enrichment cultures, isolation of a pure culture, growth of pure cultures and cell-free extracts

A heterotrophic utilizer of LAS was purified by selecting for growth with linear alkyldiphenyletherdisulphonate, dilution steps and repeated cycles of plating on LAS agarose plates, from which many colonies had to be picked, and linear alkyldiphenyletherdisulphonate liquid culture. *Delftia acidovorans* SPB1 (DSM 12586) (Schulz *et al.* 2000) and *P. lavamentivorans*[T] DS-1 (DSM 13023[T], NCIMB 13966[T]) (Schleheck *et al.* 2000) were grown as described previously.

Degradation of SP(d)Cs was examined in medium free of glass fibre. The inoculum was either *D. acidovorans* SPB1 or suspended cells from enrichment cultures able to completely degrade LAS. The latter could be subcultured repeatedly in fresh SP(d)C-medium. Cells from portions (100 ml) of outgrown cultures were harvested, washed, suspended, disrupted in a French press and used in enzyme assays as described elsewhere (Schulz *et al.* 2000).

Molecular genetic analysis

DNA was extracted from 5 g samples on polyester fleece using methods which have been shown to lyse all methanotrophs in the University of Warwick Culture Collection (McDonald and Murrell 1997). Three primer sets were used for PCR amplification of any methane monooxygenase (MMO) genes in DNA samples from the trickling filter. The first was a general primer set, A189/A682 (Holmes *et al.* 1995), which amplifies a 525-base pair (bp) internal fragment of the α-subunit of the particulate methane monooxygenase (pMMO) gene (*pmoA*) found in all methanotrophs and the homologous ammonia monooxygenase (AMO) gene of β-subclass proteobacterial

nitrifiers (*amoA*). Methanotroph-specific PCR amplification used the *pmoA*-specific primer set A189/mb661 (Costello and Lidstrom 1999) (500 bp product) and the primer set *mmoX* f882/*mmoX* r1403 (McDonald *et al.* 1995), which amplifies a 550-bp fragment of the hydroxylase component from soluble methane monooxygenase (sMMO) gene cluster. The presence of AMO genes was detected with the primer set *amoA*-IF/*amoA*-2R (Rotthauwe *et al.* 1997), which is specific for nitrifiers in *β-Proteobacteria*. The environmental *amoA* sequences have been deposited in the GenBank database under the accession numbers AF239878–AF239884.

RESULTS

Products generated from LAS by *P. lavamentivorans*^{T}

The products formed during growth of *P. lavamentivorans*^{T} with commercial LAS (C_{10}–C_{13}; 100 mg C l^{-1}) were examined by HPLC-ES-MS and grouped according to their side chain lengths (Fig. 2). In addition to some undegraded LAS, three groups of products were observed. The bulk of the products was a set of SPCs, with chain lengths C_4–C_{13}, and with C6-SPC being the most strongly represented (Fig. 2). The α,β-unsaturated SPCs (see below for identification) were detected in much lower concentrations, but over the same range of side chain lengths (C_4–C_{13}) (Fig. 2). The SPdCs, in concentrations apparently similar to the α,β-unsaturated SPCs, were detected in the sidechain lengths C_8–C_{12} (Fig. 2), but this range is probably an underestimate, because the short retention times of these tribasic anions on the reversed-phase column suggest that further representatives could have eluted with the dead volume and escaped detection. No sulphate was generated during degradation and there was no loss of u.v.-absorption, so negligible SPC was degraded. The new isolate, *P. lavamentivorans* AN-8 (see below), yielded the same SPCs and α,β-unsaturated SPCs from commercial LAS (compare Fig. 2), but apparently no SPdCs (not shown).

The SPCs formed from 3-C12-LAS by *P. lavamentivorans*^{T} in 3 days were examined by HPLC-u.v. (Fig. 3a) (inorganic buffer) and HPLC-ES-MS (Fig. 3b) (organic buffer), and their identities (e.g. Fig. 3c) were interpreted from the mass spectra as described elsewhere (Eichhorn and Knepper 2002). The starting material contained several other LAS isomers (<5% of total LAS, see 'Materials' section), all of which had disappeared when the sample was taken (Fig. 3a). The products previously detected by matrix-assisted laser-desorption-ionisation time-of-flight mass spectrometry, C6-SPC as the major product, C7-SPC and C8-SPC (Schleheck *et al.* 2000), were confirmed (peaks 3–5 in Fig. 3a). In addition, the HPLC-ES-MS method (Eichhorn and Knepper 2002) allowed 15 other related compounds to be detected: SPCs with sidechain lengths C_4–C_{11} and an α,β-unsaturated C6-SPC were found (Fig. 3b) and identified, as were four SPdCs and five other α,β-unsaturated SPCs (data not shown). The mass-spectral identification of the C6-α,β-unsaturated SPC is shown in Fig. 3c.

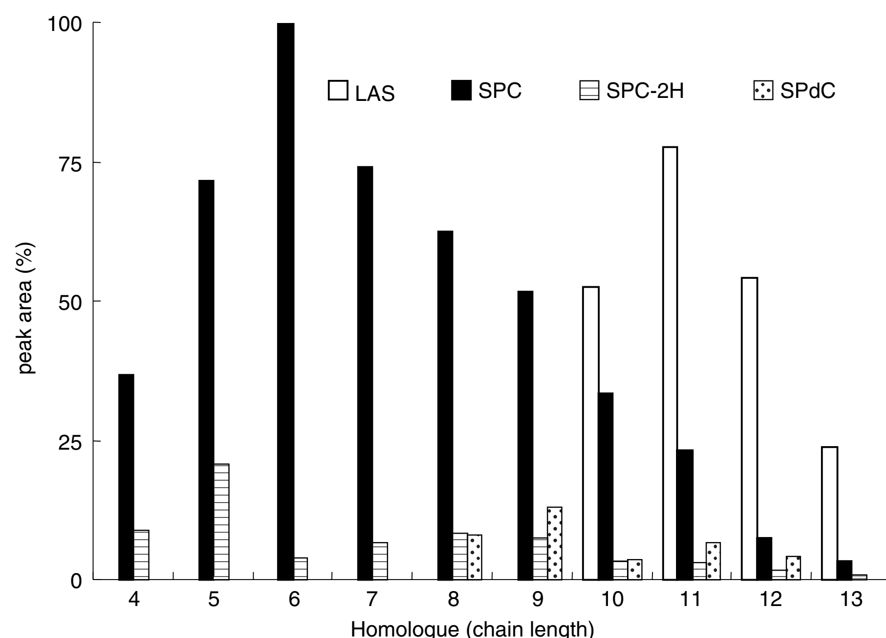


Fig. 2 Products formed during growth of *Parvibaculum lavamentivorans*^{T} with commercial linear alkylbenzenesulphonates (LAS) in salt medium. The products were separated by HPLC-ES-MS, and the data represent the compounds identified and their relative amounts. Some residual substrate (LAS) was present. The abbreviation 'SPC-2H' refers to α,β-unsaturated SPCs

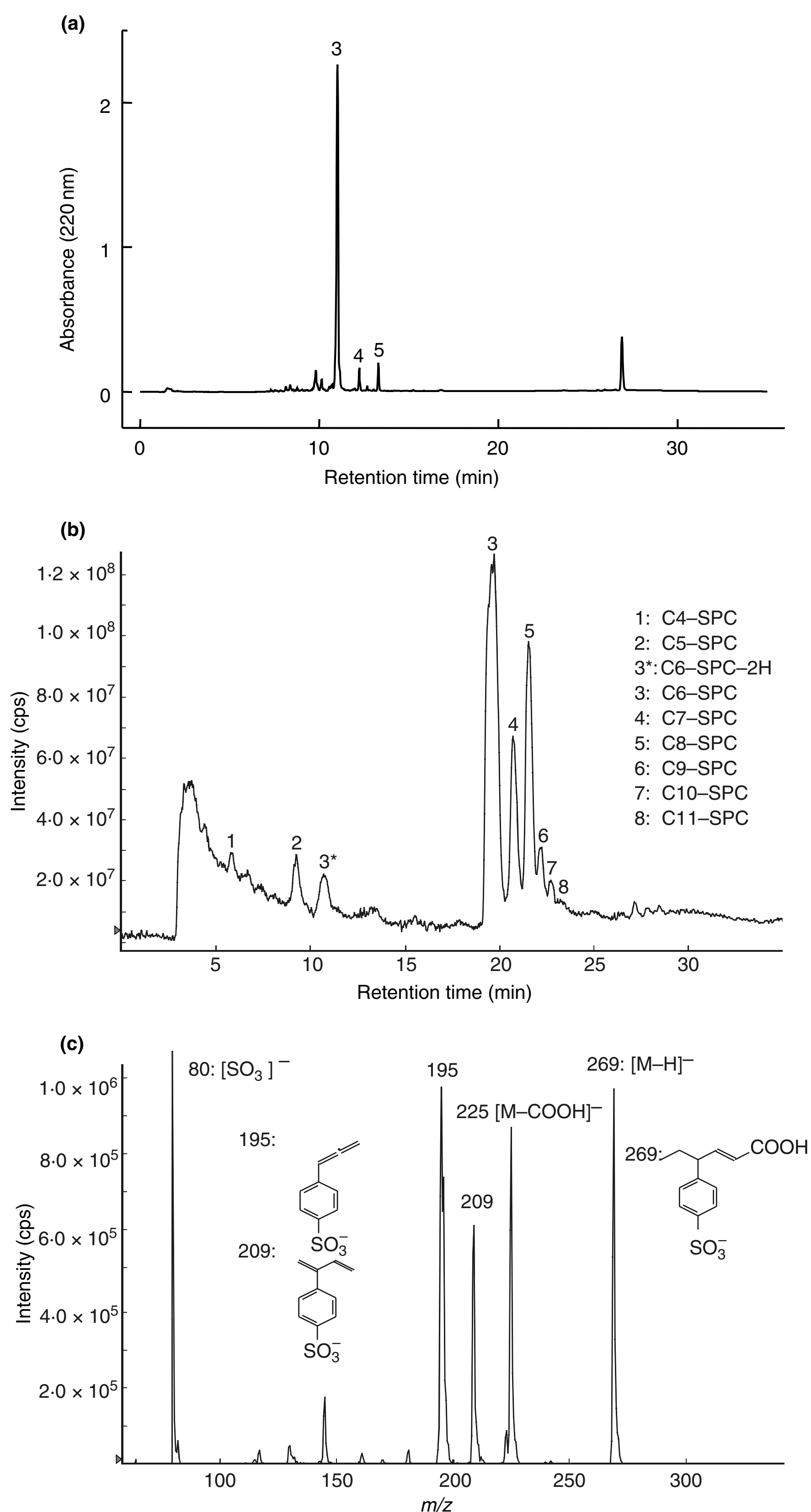


Fig. 3 HPLC-u.v. (a), HPLC-ES-MS analysis (b) of products from the degradation of 3-C12-LAS by strain DS-1 and mass spectral identification (c) of an α,β-unsaturated C6-SPC (C6-SPC-2H). Different chromatographic conditions were used for the separations in (a and b), where the detector was in part saturated, so they cannot be compared directly. The bulk of the product material eluted as one peak [peak 3 in (a)] with several peaks from minor products, and this major peak represented C6-SPC [peak 3 in (b)]. The mass spectrum of peak 3* from (b) is shown in (c)

General pattern of LAS degradation

We examined whether *P. lavamentivorans*^{T} or organisms with similar properties, were widespread in the environment, and we chose Baumann's trickling-filter model of a sewage works for the project; it represents a 6-h retention time (Baumann *et al.* 1990; Kölbener *et al.* 1995a). Individual trickling filters were prepared from inocula from six sources, three of which were pristine (littoral and 80-m profundal sediment from Lake Konstanz, and forest soil from a nature reserve) and three extensively used by man (two sewage works and an agricultural soil). The same general pattern of disappearance and transformation was seen in all cases: LAS disappeared in 3–48 days, lc-SPC had usually disappeared by day 79, and all sc-SPC species were removed by day 200. LAS (Fig. 4a) eluted from a trickling filter when the latter was new, but partial disappearance of LAS and transient appearance of putative lc-SPC (Fig. 4b) was soon observed. The centrally substituted alkyl chains of LAS (the first to elute in any set of isomers; Kölbener *et al.* 1995b) were degraded last (Fig. 4c), which represents Swisher's 'distance principle' (Swisher 1987). The transient intermediates all had u.v.-spectra indistinguishable from that of LAS and of authentic 2-C4-SPC. The chain length of the SPCs, which was observed, decreased with time (shorter retention time on the reversed phase column, Fig. 4d,e). Essentially all u.v.-absorbing compounds disappeared from the chromatogram (Fig. 4f); the residual material (Kölbener *et al.* 1996) does not concern us here.

The steady decrease in the u.v.-absorbing material leaving the trickling filter was accompanied by development of visible biomass in the trickling filter, so the disappearance of LAS was indeed biodegradation coupled to growth of microorganisms which remained attached to the fleece as biofilm (cf. Kölbener *et al.* 1995b). The peak at *ca* 2 min in, e.g. Fig. 4f, was largely nitrate (cf. Kölbener *et al.* 1995b), with occasional traces of nitrite. Each trickling filter thus contained active nitrifiers: their *amoA* genes (not shown) were typical of nitrifiers from the genus *Nitrosospira*. The detailed development of the filter varied with the inoculum, in part because it proved impossible to obtain equivalent initial biomass because of the very different characters of the samples. But for the six filters examined in any detail, the same pattern of development yielding the same stable eluate from each filter (cf. Fig. 4f) was observed. There was, thus, complete (ultimate) degradation of LAS by microbes from each inoculum.

Widespread *P. lavamentivorans*

The microbial cultures in the biofilms on the solid supports in the trickling filters, when viewed under the microscope, were complex, but each of six filters able to degrade

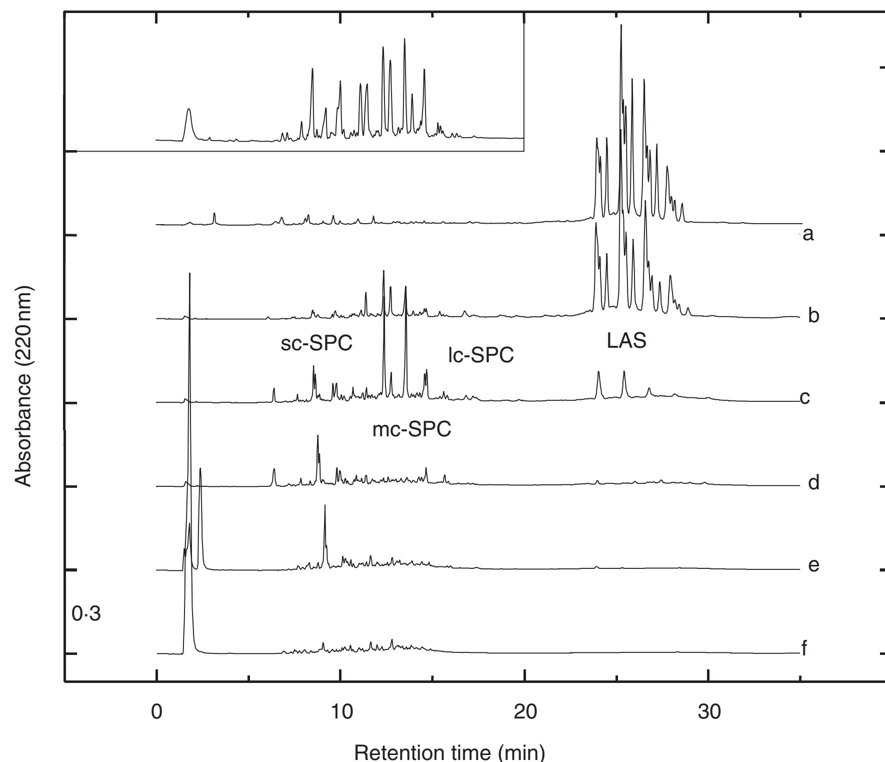


Fig. 4 Development of the degradation of commercial LAS in a trickling filter as shown by HPLC-u.v. (inorganic eluent) analyses of eluent from the filter. The inoculum was from littoral sediment from Lake Konstanz, and samples were taken at days 3 (a), 21 (b), 42 (c), 63 (d), 84 (e) and 138 (f). For comparison, a chromatogram is inset, which shows SP(d)Cs generated during growth of *Parvibaculum lavamentivorans*^{T} with commercial LAS in liquid culture

commercial LAS contained in the biofilm a significant number of organisms with the morphotype (nonmotile rod, 0·8 μm in length and 0·2 μm in diameter) of *P. lavamentivorans*^{T}. We attempted to isolate these organisms, but the three problems experienced previously, in attempts to isolate strain DS-1^{T} (Schleheck *et al.* 2000), were found to be representative. First, a solid support (fleece or glass fibre) was essential for growth in liquid culture, during which many SPCs were excreted (Fig. 2), and utilized by many other organisms. Secondly, the very slow growth of *P. lavamentivorans* on complex medium (D. Schleheck, B. Tindall, R. Rosselló-Mora and A.M. Cook, unpublished data), coupled with the complexity of the community (which rapidly overgrew strain DS-1), prevented a classical isolation. And thirdly, although the primary degrader presumably grew to pinpoint colonies on LAS agarose plates, the size of the community, which grow with SPC-products, presumably prevented these colonies from being seen: we believe that *P. lavamentivorans* (strain D in Fig. 1) obtains less carbon (e.g. four atoms in Fig. 1) and energy from LAS molecules than the SPC-degrading strains from SPC molecules (e.g. 12 atoms in Fig. 1), and that strain D does not dominate the culture numerically. An attempt was made to overcome this problem by replacing LAS (and the production of SPCs) with an analogue, linear alkyldiphenyletherdisulphonate, the products from which are not degraded under these conditions (Schleheck *et al.* 2000, 2003). Only two of the six cultures grew, one poorly and one pure culture was obtained. This grew in LAS salts glass fibre medium and excreted SPCs (see above). The organism was termed strain AN-8 and was found to be a strain of *P. lavamentivorans* (D. Schleheck, B. Tindall, R. Rosselló-Mora and A.M. Cook, unpublished data).

Ring cleavage reaction and absence of methanotrophs

Delftia acidovorans SPB1, the only organism with a known ring cleavage reaction in the degradation of an SPC, has a narrow substrate range (Schulz *et al.* 2000) and showed insignificant growth with 4-C6-SPC and with SP(d)Cs (the products in Fig. 2). In contrast, suspended cells (no glass fibre) from each of the six trickling filters could be repeatedly subcultured in SP(d)C salt medium; the aromatic compounds were degraded (HPLC), and cell material and sulphate were recovered. Extracts of cell material from one representative culture were examined and they contained 4-sulphocatechol 1,2-dioxygenase with a specific activity of *ca* 0·7 mkat (kg protein)$^{-1}$, whereas the pure culture of *D. acidovorans* SPB1 has a specific activity of 0·3 mkat (kg protein)$^{-1}$. It is inferred that the *ortho* cleavage of 4-sulphocatechol is the normal pathway through which intermediates of LAS are channelled.

The only defined bacterial community in which commercial LAS is degraded, involves a methanotroph which attacks LAS only in the presence of methane (Hrsák and Begonja 1998, 2000) (Fig. 1), so we assayed for methanotrophs in a representative trickling filter. No PCR amplification products were obtained with the primer sets that are specific for pMMO or sMMO genes. Methanotrophs thus played no significant role in these experiments, as might be anticipated from the high level of aeration of the system and the correspondingly low pCO_2 which precluded growth of methanogens in the thick biofilm.

DISCUSSION

Schleheck *et al.* (2000) wondered whether the pure culture, now *P. lavamentivorans*^{T} (D. Schleheck, B. Tindall, R. Rosselló-Mora and A.M. Cook, unpublished data), attacked LAS by ω-oxygenation and β-oxidation, or by subterminal monooxygenation followed by a Baeyer–Villiger monooxygenation to an ester, whose cleavage would also yield a SPC from the relevant LAS congener. The α,β-unsaturated SPCs of all chain lengths (Fig. 2) are the extracellular intermediates which prove the activity of β-oxidation at all chain lengths. The intracellular intermediates, however, would be CoA esters, as the biochemical abstraction of the β-hydrogen atom from the carboxylate (at neutral pH in aqueous solution at 30°C) to generate the α,β-unsaturated compound depends on the activation by an CoA substituent and on acyl-CoA dehydrogenase (e.g. Lehninger *et al.* 1993; Metzler 2001).

The enzymes of β-oxidation usually have broad optimal activity in ranges of chain lengths accepted (Lehninger *et al.* 1993), and strain DS-1 (with strain AN-8) obviously has low activity with C6-SPC-CoA, which is deesterified and excreted (Fig. 2). We also infer that β-oxidation is hindered when the 4-sulphophenyl substituent comes into the vicinity of the CoA-ester. These large CoA-ester intermediates, none of which has been observed experimentally, will not diffuse out of the cell ($M_r > 100$; e.g. Madigan *et al.* 2003), so we postulate a very active thioesterase within the cell, which acts on the range of short-chain CoA intermediates prior to excretion, whether in native samples (e.g. Eichhorn and Knepper 2002) or in pure cultures (Figs 2 and 3c). The nature of the export of these many polar compounds from the cell remains to be explored.

The data in Fig. 2 also show that C13-LAS is subject to oxidation to C13-SPC. This can only happen via an ω-oxygenation. No evidence for α-oxidation was found, so the C12-SPCs (Fig. 3) also arose from ω-oxygenation. It remains to be seen which of about five known monooxygenase (Powlowski and Shingler 1994; Murrell and Holmes 1996) or dioxygenase (Maeng *et al.* 1996) mechanisms is involved, and which families of alcohol and aldehyde dehydrogenases generate the lc-SPC.

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Detailed analysis of the products obtained during growth of strain DS-1 with 3-C12-LAS (and traces of other congeners) confirms, and expands on, this picture. The short-chain intermediate which was generated in large amounts, 4-C6-SPC, with the C8- and C10-SPCs and a trace of C4-SPC, together with the α,β-unsaturated C6-SPC, that was detected (Fig. 3b,c), support only ω-oxygenation, oxidation and β-oxidation. Similarly, we interpret the generation of some C7-SPC, less C9- and C11-SPCs and a trace of C5-SPC (Fig. 3b), together with α,β-unsaturated C7-SPC (not shown), as products from the trace of 3-C11-LAS present in the starting material. The trace of 5-C11-LAS was presumably the source of the C7-SPdC, which was also detected (not shown).

Several products (about four) are thus generated from any one LAS congener (Fig. 3b), a fact which is masked when the commercial product is used (Fig. 2). The span of products from commercial LAS is similar to those from 3-C12-LAS, except that many more SPdCs are formed (Figs 2 and 3b); the essential difference between 3-C12-LAS (with traces of other congeners) and commercial LAS is the large number of centrally substituted LAS species in the commercial product. Presumably the 2- and 3-substituted LAS congeners are subject only to a single ω-oxygenation (see also Hrsák and Begonja 1998), whereas some centrally substituted LAS congeners (e.g. 5-C11-LAS) can be subject to two ω-oxygenations. Many of these products (Figs 2 and 3b) are seldom observed in native samples, e.g. C12-SPC (González-Mazo *et al.* 1997) or SPdCs (di Corcia *et al.* 1999), but the chemicals are stable. This presumably indicates how readily these compounds are metabolized in nature.

The data from the trickling filters (Fig. 4) indicate that only one set of extracellular intermediates is found during the degradation of LAS, SP(d)Cs, many of which can be α,β-unsaturated (Figs 2 and 3b). This observation is valid for samples from pristine and contaminated environments, and it expands both the work with OECD tests, which do not measure intermediates (e.g. Kölbener *et al.* 1995b), and the work with chemical determinations in native environments (e.g. di Corcia *et al.* 1994, 1999; Matthijs *et al.* 1997). These intermediates can be generated by several metabolic groups of organisms, e.g. by the methanotrophs (Hrsák and Begonja 1998), possibly by the ammonia oxidizers (Brandt *et al.* 2001), and by heterotrophic organisms (Fig. 1). The latter make the major contribution under our conditions, where methanotrophs could not be detected. Inhibition of ammonia monooxygenase with thiourea (W. Dong, unpublished data) had only a minor effect on the degradation of LAS, so ammonia oxidizers play at best a small role in LAS degradation. The degradation of LAS in these trickling filters is thus carried out essentially entirely by heterotrophs.

Eichhorn and Knepper (2002) not only used LC-MS but also GC-MS to examine their trickling filters. They found no intermediates that would indicate direct desulphonation of LAS (see Campos-García *et al.* 1999). Recent data from soil mesocosms also shows only SPCs (Elsgaard *et al.* 2003), although the authors were looking for the direct desulphonation pathway (see Kertesz *et al.* 1994). One pathway, that/those in Fig. 1, obviously dominates in the degradation of LAS in native samples and pure cultures.

The final steps in the pathway (Fig. 1) involve the *ortho* cleavage of 4-sulphocatechol. This established pathway (e.g. Feigel and Knackmuss 1993; Contzen *et al.* 2001) is found in the one characterized organism able to degrade a sc-SPC, 2-C4-SPC (strain C in Fig. 1) (Schulz *et al.* 2000), and data from our laboratory indicate its presence in cultures able to degrade 3-C4-SPC and 4-C6-SPC (D. Schleheck, unpublished data). It was possible to generate enough cell material of one mixed culture able to degrade the SPCs shown in Fig. 2 and observe that the *ortho* cleavage enzyme, which is usually inducible (Feigel and Knackmuss 1993; Schulz *et al.* 2000), is very active. 4-Sulphocatechol 1,2-dioxygenase is presumably the major ring cleavage enzyme for intermediates from commercial LAS. The nature of the enzyme (or pathway), which yields 4-sulphocatechol from each sc-SPC, is still unknown.

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