Biodegradation of synthetic surfactants: linear alkylbenzenesulfonates (LAS) and related compounds

Dissertation

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by

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für meinen Sohn Janek

„Wenn du dir ein Haustier zulegen willst, so bedenke:
Ein Hund betrachtet dich als Familienmitglied,
ein Bakterium betrachtet dich als Personal.“

Aus den USA, verändert.

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CONTENTS

ABBREVIATIONS

ABS	branched-chain alkylbenzenesulfonate surfactant
CoA	coenzyme A
DSDPEC	disulfodiphenylether carboxylates
DSM	Deutsche Sammlung für Mikroorganismen
GC	gas chromatography
HPLC	high performance liquid chromatography
IC	ion chromatography
IR	infra red
LC-MS	liquid chromatography mass spectroscopy
LADPEDS	linear monoalkyldiphenyletherdisulfonate surfactant
LAS	linear alkylbenzenesulfonate surfactant
MALDI-TOF	matrix-assisted laser desorption ionisation time of flight
MSDPEC-phenol	monosulfodiphenylether carboxylate-phenol
ND	not detected
n.d.	not determined
NMR	nuclear magnetic resonance
OD	optical density
PCR	polymerase chain reaction
SPdC	sulfophenyldicarboxylates
SP(d)C	mixture of sulfophenylcarboxylates and sulfophenyldicarboxylates
SPC	sulfophenylcarboxylates
TLC	thin layer chromatography
tr	trace amount
UV	ultraviolet

SUMMARY

Commercial linear alkylbenzenesulfonate (LAS) surfactant is the major, xenobiotic compound released into the environment, and is completely biodegraded under oxic conditions, e.g. in sewage-treatment plants, as has been known for more than 40 years. The first representative, heterotrophic organism, a bacterium, proven to utilise LAS was not reported until 2000. Strain DS-1^T catalyses the ω-oxygenation of the LAS sidechain and spirals of ß-oxidation, and a wide range of sulfophenylcarboxylates (SPCs), and related compounds, is formed from commercial LAS, which nominally comprises 20 compounds. Other organisms mineralise these SPCs. In this thesis, the microbiology of the bacterial community, the analytical chemistry, and the biochemistry involved in LAS-degradation were further explored.

Strain DS-1^T utilised LAS and many other anionic and nonionic surfactants concomitant with formation of a biofilm. The organism also grew with e.g. acetate or octane, but required no biofilm. Strain DS-1^T was indicated as type strain of a species in a new genus within the α-subclass of Proteobacteria, based on 16S-rDNA sequence comparison, and analysis of the lipid components. The name *Parvibaculum lavamentivorans*^{T} was proposed.

Strain DS-1^T displayed activity of the LAS-degradative enzymes during growth with LAS and during growth with acetate. Specific LAS-dependent oxygen uptake, concomitant with substrate degradation and formation of SPC, was measurable in whole cells, but not in cell-extracts. Many surfactants, or octane, caused oxygen uptake. Cell suspensions did not attack LAS in absence of molecular oxygen, or when pretreated with the cytochrome P450-specific inhibitor metyrapone in presence of molecular oxygen. Absorption maxima indicative of a cytochrome P450-like protein present in the soluble fraction of crude extract where detected. A soluble, P450-type oxygenase system, which catalyses ω-oxygenation of LAS in strain DS-1^T is inferred.

Strain DS-1^T utilised linear alkyldiphenyletherdisulfonate (LADPEDS) surfactant, a complex mixture, for growth, and formed characterised disulfodiphenylether carboxylates (DSDPECs). DSDPECs were not degraded when supplied as carbon source for bacteria from activated sludge, but DSDPECs were subject to quantitative desulfonation when supplied as sole source of sulfur for growth of an isolate obtained, *Rhodococcus opacus* ISO-5. On average, one sulfonate group was removed per DSDPEC species, and largely monosulfodiphenylethercarboxylate-phenols (MSDPEC-phenols) generated. MSDPEC-phenols were subject to extensive dissimilation by bacteria from activated sludge.

Strain DS-1^T degraded the laterally-substituted, LAS congener 2-(4-sulfophenyl)dodecane (2-C12-LAS) largely to 3-(4-sulfophenyl)butyrate (3-C4-SPC), 3-C12-LAS largely to 4-C6-SPC, and 2-C11-LAS largely to 4-C5-SPC. Traces of many other SPCs were detected, e.g. α,β-unsaturated SPC-species (SPC-2H). *Comamonas testosteroni* strains SPB-2 and KF-1, which utilised 3-C4-SPC, were isolated, as was *Delftia acidovorans* SPH-1, which utilised 4-C6-SPC enantioselectively. The SPC-degradative pathways apparently involved 4-sulfocatechol, and inducible 4-sulfocatechol-1,2-dioxygenase. Substrate-dependent oxygen uptake of whole cells of *C. testosteroni* indicated inducible oxygenation of 3-C4-SPC, and of 4-sulfophenol, in cultures grown with 3-C4-SPC, or 4-sulfophenol. 4-Sulfophenol was inferred as an intermediate of 3-C4-SPC degradation. Growth of strain DS-1^T in community with strain SPB-2 (or strain KF-1) and strain SPH-1 with commercial LAS indicated, that each new isolates had only a narrow substrate range for SPC. This community mineralised four major SPCs derived from eight of the 20 LAS congeners and allowed the prediction that 11 major SPCs are generated from the degradation of all the 20 congeners of commercial LAS. Thus, many more organisms are needed to degrade commercial LAS fully.

Strain DS-1^T degraded the centrally-substituted, LAS congener 5-C10-LAS largely to 4-C8-SPC (and minor SPCs), and to sulfophenyldicarboxylates (SPdC) of chain lengths of C6, C8, and C10. The degradation of 6- and 5-C12-LAS yielded largely C8-SPC species, and C6-, C8-, C10-, and C12-SPdC. A preparation of 7-, 6- and 5-C13-LAS was degraded to C9- and C7-SPCs, and to C5-, C7-, C9-, C11- and C13-SPdCs. This set of data confirmed the generation of the 11 predicted SPCs, and that SPdCs are only generated during degradation of the centrally-substituted LAS congeners by strain DS-1^T. The range of products generated from commercial LAS includes 11 major SPCs, each with one minor SPC and SPC-2H-species, as well as about 17 SPdCs. Thus, the range of products generated from commercial LAS by strain DS-1^T is more complex than previously realised.

ZUSAMMENFASSUNG

Das handelsübliche Tensid Lineares-Alkylbenzolsulfonat (LAS) ist die mengenmäßig bedeutendste xenobiotische Verbindung, die in die Umwelt freigesetzt wird. LAS wird unter oxischen Bedingungen vollständig biologisch abgebaut, z.B. in Kläranlagen. Obwohl dies seit 40 Jahren bekannt ist, gelang die Isolierung eines repräsentativen Mikroorganismus, der LAS für sein heterotrophes Wachstum nutzen kann, erst im Jahr 2000. Dieser Stamm, DS-1^T, greift die LAS-Alkylseitenkette durch ω-Oxygenierung und β-Oxidation an, wobei aus den 20 Einzelverbindungen des handelsüblichen LAS viele verschiedene Sulfophenylcarboxylate (SPC) und ähnliche Verbindungen gebildet werden. Diese SPCs werden von anderen Organismen vollständig abgebaut. In dieser Arbeit wurde die Mikrobiologie der bakteriellen Gemeinschaft, sowie die chemische Analytik und die Biochemie des LAS-Abbaus weiter erforscht.

Stamm DS-1^T kann LAS und viele weitere anionische und nichtionische Tenside zum Wachstum nutzten, wobei er Biofilm bildet. Bei Wachstum mit Acetat oder Oktan wird kein Biofilm gebildet. Basierend auf 16S rDNA Sequenzvergleichen und der Analyse der Membrankomponenten wurde Stamm DS-1^T als Typ-Stamm einer neuen Art in einer neuen Gattung innerhalb der α-Proteobakterien eingeordnet und der Name *Parvibaculum lavamentivorans*^{T} vorgeschlagen.

Die Enzyme des LAS-Abbauwegs in Stamm DS-1^T waren während des Wachstums mit LAS und während des Wachstums mit Acetat aktiv. In Zellsuspensionen war spezifische LAS-abhängige Sauerstoffaufnahme meßbar, einhergehend mit LAS-Abbau und SPC-Bildung, nicht aber in Zellextrakten. Viele weitere Tenside und auch Oktan führten zu einer Sauerstoffaufnahme. Zellsuspensionen bauten LAS in Abwesenheit von molekularem Sauerstoff nicht ab und nicht in Anwesenheit von molekularem Sauerstoff, wenn die Zellen mit dem Cytochrom-P450-spezifischen Inhibitor Metyrapone vorbehandelt waren. Die lösliche Fraktion des Zellextrakts zeigte Absorptionsmaxima spezifisch für ein Cytochrom-P450-ähnliches Protein. Es wird vermutet, dass die ω-Oxygenierung von LAS in Stamm DS-1^T durch ein lösliches, P450-abhängiges Oxygenasesystem katalysiert wird.

Stamm DS-1^T wuchs mit dem Tensid Lineares-Alkyldiphenyletherdisulfonat (LADPEDS), ein komplexes Gemisch, und bildete viele Disulfodiphenylethercarboxylate (DSDPEC). DSDPEC als Kohlenstoffquelle wurden durch Bakterien aus Klärschlamm nicht weiter abgebaut. Jedoch nutzt ein Neuisolat, *Rhodococcus opacus* ISO-5, DSDPEC als Schwefelquelle und desulfoniert diese quantitativ. Durchschnittlich wurde eine Sulfonatgruppe pro DSDPEC-Verbindung entfernt und

größtenteils Monosulfodiphenylethercarboxylat-Phenole (MSDPEC-Phenole) gebildet. MSDPEC-Phenole unterlagen einem weitgehenden Abbau durch Bakterien aus Klärschlamm.

Die lateral-substituierte LAS-Verbindung 2-(4-Sulfophenyl)dodekan (2-C12-LAS) wurde von Stamm DS-1^T überwiegend zu 3-(4-Sulfophenyl)butyrat (3-C4-SPC) abgebaut, sowie 3-C12-LAS überwiegend zu 4-C6-SPC, und 2-C11-LAS überwiegend zu 4-C5-SPC. Viele weitere SPC-Nebenprodukte wurden gebildet, z.B. α,β-ungesättigtes SPC. Es konnten Stämme isoliert werden, die 3-C4-SPC abbauen, *Comamonas testosteroni* SPB-2 und KF-1, sowie ein Stamm, der 4-C6-SPC enantioselektiv abbaut, *Delftia acidovorans* SPH-1. Der SPC-Abbau erfolgt offensichtlich über 4-Sulfocatechol und über induzierbare 4-Sulfocatechol-1,2-Dioxygenasen. Substratabhängige Sauerstoffaufnahme durch ganze Zellen der *C. testosteroni* Stämme während des Wachstums mit 3-C4-SPC oder mit 4-Sulfophenol, deutete auf induzierbare Oxygenierung von 3-C4-SPC und von 4-Sulfophenol hin. Es wird vermutet, dass 4-Sulfophenol ein Intermediat des 3-C4-SPC-Abbauwegs ist. Das Wachstum von Stamm DS-1^T in Gemeinschaft mit Stamm SPB-2 (oder KF-1) und Stamm SPH-1 mit handelsüblichem LAS deutete darauf hin, dass jedes der neuen Isolate nur ein enges Substratspektrum für SPC besitzt. Diese Gemeinschaft baut vier SPC-Hauptprodukte, gebildet aus acht von 20 LAS-Einzelverbindungen, vollständig ab, wobei insgesamt vermutlich 11 SPC-Hauptprodukte aus allen 20 Verbindungen entstehen. Somit sind sehr viel mehr Organismen notwendig, um handelsübliches LAS vollständig abzubauen.

Die zentral-substituierte LAS-Verbindung 5-C10-LAS wurde von Stamm DS-1^T überwiegend zu 4-C8-SPC (und Nebenprodukte) abgebaut, sowie zu Sulfophenyldicarboxylaten (SPdC) der Kettenlängen C6, C8 und C10. Der Abbau von 6- und 5-C12-LAS führte überwiegend zu C8-SPC-Verbindungen, sowie zu C6-, C8-, C10- und C12-SPdC-Verbindungen. Eine Präparation aus 7-, 6- und 5-C13-LAS wurde zu C9- und C7-SPC-Verbindungen, sowie zu C5-, C7-, C9-, C11-, und C13-SPdC-Verbindungen, abgebaut. Diese Daten bestätigen, dass handelsübliches LAS von Stamm DS-1^T zu 11 SPC-Hauptprodukten abgebaut wird, und dass SPdC nur im Zuge des Abbaus der zentral-substituierten LAS-Verbindungen entsteht. Die Produkte umfassen somit 11 SPC-Hauptprodukte, jeweils ein SPC- und SPC-2H-Nebenprodukt, sowie bis zu 17 verschiedene SPdC-Verbindungen. Das Spektrum der Produkte, das aus handelsüblichem LAS durch Abbau von Stamm DS-1^T gebildet wird, ist somit weit umfangreicher als ursprünglich angenommen.

CHAPTER 1

General Introduction

Spiegel Nr. 30 / 7/2003

BRASILIEN

Barfuß durch die Brühe

In einem der größten industriellen Ballungszentren der Welt spielt sich eine Umwelt-Katastrophe apokalyptischen Ausmaßes ab.

Weiße Flocken tanzen über dem Hauptplatz von Pirapora do Bom Jesus. Mit jeder Windböe steigen sie aus dem Fluss Tietê auf, der durch die Kleinstadt 54 Kilometer nordwestlich von São Paulo fließt. Sie wirbeln durch die Straßen, verfangen sich in den Palmen vor dem Rathaus, bedecken Häuser und Autos.

Daniela Araújo, 17, schließt alle Türen und Fenster und zieht sich mit ihren Geschwistern ins Hinterzimmer ihrer Hütte zurück. Der Schaum, der aus dem Fluss quillt und ihre Veranda einhüllt, ist pures Gift: Er hinterlässt einen schwarzen Schmierfilm aus Schwermetallen und Fäkalien an Wänden und Möbeln, verätzt Elektrogeräte, zerfrisst den Lack der Autos.

Lange bevor der Fluss Tietê Pirapora erreicht, fließt er durch São Paulo. Dort nimmt er die Abwässer von mehr als 17 Millionen Einwohnern auf. Vor dem Städtchen stürzt die Jauche dann über eine 25 Meter hohe Staustufe. „Der Damm funktioniert wie eine gigantische Waschma-

(...)

FOTOS: HELGO FOSMER / AE

Abwasserrückstände in Pirapora: *Kloake mitten in der Stadt*

Schaumwand in einer Straße, Anwohnerin *Fußnägel verloren*

Schadstoffe im Wasser hat stark zugenommen. Fäkaliengeruch kündet schon zehn Kilometer vor Pirapora von dem Umweltdesaster. Wie eine Schneelawine schiebt sich der schaumbedeckte Fluss durch die grüne Landschaft. Viele der 15 000 Einwohner leiden an Asthma und Hautkrankheiten. In der einzigen Gesundheitsstation werden dreimal so viele Patienten versorgt wie sonst. Acht von zehn behandelten Kindern haben Atemprobleme.

Trotz der Verschmutzung haben viele arme Familien ihre Hütten direkt am Flussufer errichtet. Kinder baden und spielen in

(...)

BIODEGRADATION OF SURFACTANTS

The ancient Sumerians published the first known synthesis of a surfactant about 4500 years B.P. (before the present), natural, biodegradable soap derived from saponification of fat with soda (Jakobi and Löhr 1987). Soap remained as a luxury for the next 4300 years, regarded as medication and as a cosmetic rather than as the washing agent we know today. This only changed with the development of practical means for the large-scale production of soda. At the beginning 20th century, the first commercial detergent formulation (`Persil´, 1907) for the routine, manual washing of textiles was introduced, with soap as the `surface-acting´ (surfactant) ingredient (Wagner 2001).

Soap in particular, and surfactants in general, are water-soluble amphiphiles, containing one (or more) non-polar, hydrophobic moiety (usually a long alkyl chain) attached to one (or more) hydrophilic, solubility-enhancing moiety (Fig. 1). In aqueous solution, they support the washing process through enhanced removal of poorly water-soluble impurities (`soil´) from a solid, and by dispersing the soil in the wash liquor.

The changes required when machine-washing was introduced, especially problems with water hardness, caused that soap was replaced gradually by synthetic surfactants. The first practical substitute for soap was fatty-alcohol sulfate (`Fewa´, 1932). In the 1950´s, new, stable surfactants were introduced by the petrochemical industry, especially tetrapropylene-benzenesulfonate (Fig. 1). The high washing power, widespread availability, and low price, led to these surfactants meeting about 50 % of the surfactant-demand in the Western world (Jakobi and Löhr 1987; Schulze 1996).

It was then that the biodegradability of surfactants was discovered to be an important, additional criterion for evaluating these products. Insufficient biodegradation led to the development of great masses of foam in streams and rivers in vicinity of dams or other obstructions (see opposite page), e.g. in Germany in the 1960´s (Jakobi and Löhr 1987).

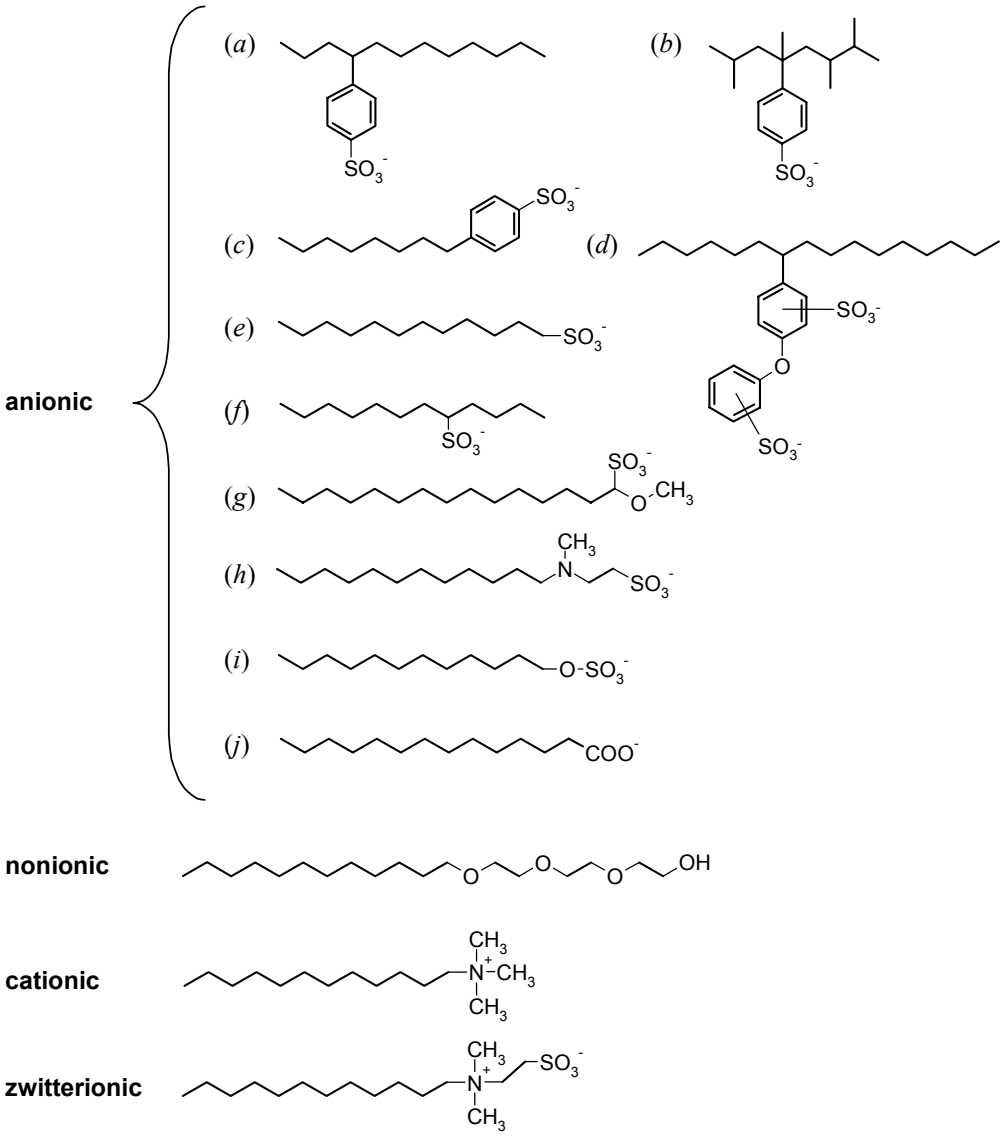

FIG. 1. Illustrative examples of surfactants. Surfactants are classified according to the nature of the charge of their polar moiety into anionic, cationic, zwitterionic (amphoteric), and nonionic surfactants, and many other smaller categories (Hoffmann and Ulbricht 1993; Wagner 2001). Anionic surfactants are the major class on a world-wide tonnage basis, and linear alkylbenzenesulfonate (LAS) is the most important anionic surfactant (2.5 million tonnes per year, Schulze 1996): in average, 3 g of LAS is used per person and day (http://www.lasinfo.org), and LAS is thus the major xenobiotic compound which is released into wastewater-treatment plants.

Anionic surfactants: *a*, linear alkylbenzenesulfonate (LAS); *b*, branched-chain alkylbenzenesulfonate (ABS) [tetrapropylenebenzenesulfonate]; *c*, 4-(1-*n*-octyl)benzenesulfonate (OBS); *d*, linear hexadecanediphenyletherdisulfonate (LADPEDS); *e*, 1-alkanesulfonate; *f*, secondary alkanesulfonate; *g*, methylestersulfonate; *h*, alkylmethyltaurate; *i*, fatty-acid sulfate; *j*, soap. Representatives of the nonionic, cationic, and zwitterionic surfactants are alkylethoxylate, alkyltrimethylammonium, and alkylsulfobetaine, respectively.

The discovery, that surfactants could pass essentially undegraded through modern wastewater-treatment plants, and thus enter the surface waters, led to both legislation and voluntary agreements between industry and government, at least in Western countries, which effected the transition to the use of biodegradable surfactants in household detergents. In Germany by 1964, the branched-chain alkylbenzenesulfonate (ABS) surfactants, e.g. tetra-propylenebenzenesulfonate (Fig. 1), which resisted biodegradation, were replaced by linear alkylbenzenesulfonate (LAS) surfactants, which, if pure, are completely biodegradable (Sawyer and Ryckman 1957; Swisher 1987; Schöberl 1989; Kölbener *et al.* 1995a).

From the microorganisms´ viewpoint, surfactants represent a potential source of carbon and energy for heterotrophic growth, despite the fact that these chemicals can be toxic. Bacteria use essentially two strategies to access the carbon in surfactants (Swisher 1987; White and Russell 1994), the bulk of which (at least in ionic surfactants) is generally present in the hydrophobic moiety (i.e. the alkyl chain, Fig. 1). The first strategy involves an initial separation of the hydrophile from the hydrophobe (hydrophile attack), which is then oxidatively degraded. In the second mechanism, the hydrophobe is initially oxidised while still attached to the hydrophile (hydrophobe attack, Fig. 2). Both strategies lead to immediate loss of amphiphilicity in the molecule, which therefore no longer behaves as a surfactant.

Residues of this primary degradation of surfactants (Fig. 2) may still contain much carbon to support microbial growth. The subsequent breakdown of these residues to biomass H_2O, CO_2, and mineral salts, represents the complete degradation of surfactants (mineralization).

For both mechanisms, hydrophile attack and hydrophobe attack, the oxidation of the alkyl-chain hydrophobe follows the pathway of chain-shortening through fatty-acid β-oxidation, and for the second mechanism, the surfactant molecule has to be initially activated as corresponding fatty-acid derivative, *via* ω-oxygenation and oxidations (Fig. 2). The extensive methyl-branching of the alkyl chain of ABS hinders these reactions, and explains the slow disappearance of ABS from the environment.

BIODEGRADATION OF LAS

LAS is removed to about 99.9 % by a functional sewage treatment plant, largely through biodegradation (e.g. Schöberl 1997). Short-chain sulfophenylcarboxylate (SPC) residues appear as transient intermediates after the first degradative step (Fig. 2), which involves ω-oxygenation, oxidation, and β-oxidation. SPCs are then completely degraded in the second degradative step, by other organisms, through ring-opening and desulfonation (Swisher 1987; Schöberl 1989; White and Russell 1994).

Exhaustive work has gone into confirming the complete biodegradability of LAS (e.g. Schöberl 1997), and though these data allow a thorough ecological risk assessment (e.g. HERA 2002), we still know as little about the degradation processes and the microbial communities involved as we did when Swisher first published 30 years ago (Swisher 1970).

The supposedly facile degradation of LAS is more complex than previously realised. LAS is not a single compound, but, ideally, a mixture of 20 compounds, all subterminally substituted, linear, alkyl chains (C_{10}-C_{13}) carrying a 4-sulfophenyl moiety. Of these 20 compounds, 18 are optically active, so there are 38 structures in the ideal mixture. The reason for this mixture involve solubility, environmental factors and production techniques, which, together with information of impurities can be found elsewhere (e.g. Kosswig 1994; Kölbener *et al.* 1995a; Kölbener *et al.* 1995b; HERA 2002). Thus, many SPCs, and similar compounds, are formed from commercial LAS, and subsequently mineralised in the second degradative step by specialised organisms (Jiménez *et al.* 1991; Sigoillot and Nguyen 1992; Hrsák and Begonja 1998).

More complex still is the mixture of compounds in commercial linear hexadecane-diphenyletherdisulfonate (LADPEDS) surfactant (Fig. 1). LADPEDS is initially degraded to short-chain residues, but these are resistant to fast biodegradation (Quencer and Loughner 2001).

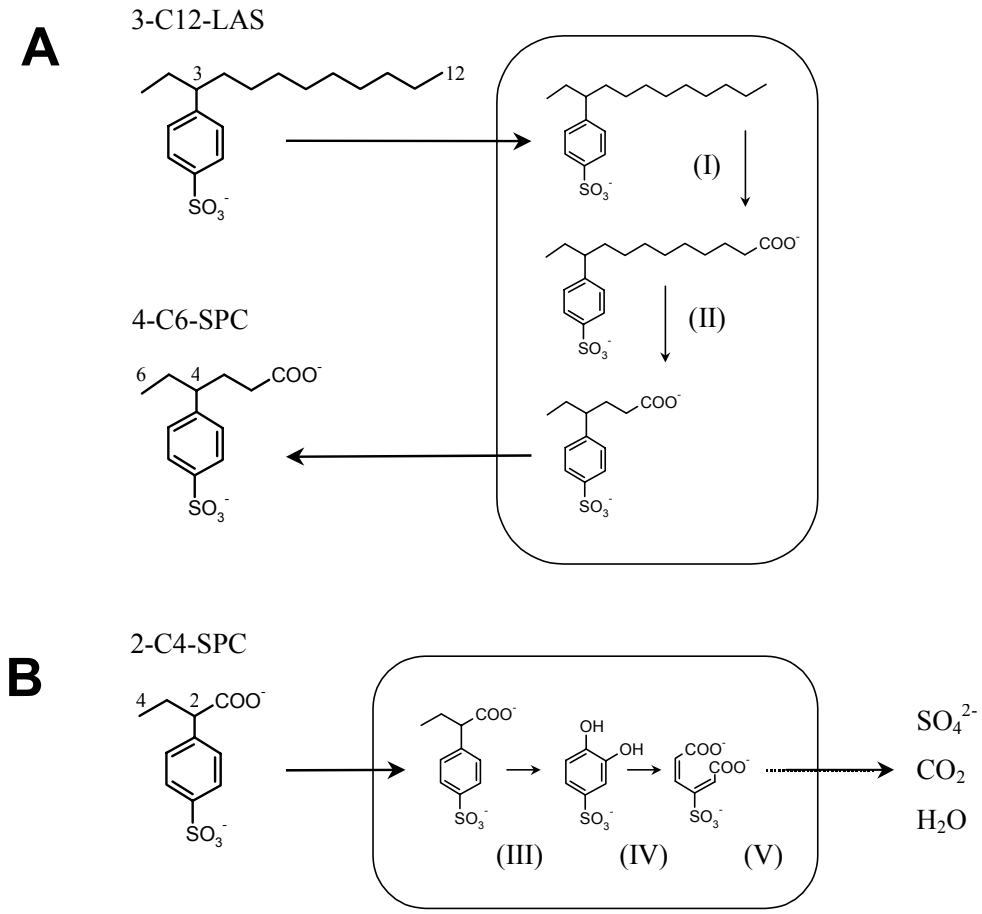

FIG. 2. Biodegradation of LAS in two tiers. The illustration shows known or anticipated LAS-degradative reactions, representative for the degradative pathway observed in nature (Dong *et al.* 2003). The reactions are catalysed by bacteria isolated in our laboratory, (A) α-proteobacterium strain DS-1 (Schleheck *et al.* 2000) (now *Parvibaculum lavamentivorans*^{T}, see Chapter 2), and (B) *Delftia acidovorans* SPB1 (Schulz *et al.* 2000). Strain DS-1^T degrades a representative LAS congener, 3-(4-sulfophenyl)dodecane (3-C12-LAS), to 4-(4-sulfophenyl)hexanoate (4-C6-SPC) *via* ω-oxygenation and oxidation (I), and chain shortening through β-oxidation (II) (Schleheck *et al.* 2000; Dong *et al.* 2003); the congeners of commercial LAS are degraded to an array of products, mainly SPCs (C4-C9) (Dong *et al.* 2003). Strain SPB1 was proven to mineralise one SPC, 2-(4-sulfophenyl)butyrate (2-C4-SPC), involving side-chain removal and dioxygenation to 4-sulfocatechol (III), 4-sulfocatechol *ortho*-cleavage (IV) (Schulz *et al.* 2000), and an *ortho*-degradation pathway (V) (Feigel and Knackmuss 1993). Nevertheless, strain SPB1 utilises none of the SPCs which are generated by strain DS-1^T during degradation of commercial LAS (Schulz *et al.* 2000).

AIM OF THIS THESIS

Up till now, no detail in any reaction of LAS degradation has been available. The interest is, thus, not whether LAS is degraded, but under which conditions, and how.

The prerequisite for this renewed attempt to understand LAS degradation was the isolation of *Parvibaculum lavamentivorans*^{T} DS-1. This organism catalyses the initial degradation of commercial LAS (Fig. 2) in pure culture under laboratory conditions (Schleheck *et al.* 2000).

Furthermore, new methods of analytical chemistry, HPLC with improved gradient systems (Matthijs and De Henau 1987; Kölbener *et al.* 1995a), and the coupling of HPLC with improved gradient system to mass spectroscopy (Eichhorn and Knepper 2002), allowed for the first time the separation, quantification, and identification of individual compounds of commercial LAS, and of the generated SPCs.

RESEARCH DONE IN COOPERATION

The results in this thesis include fruitful cooperations:

CHAPTER 2. The chemotaxonomic analysis of lipids in strains DS-1^T and JP57 was done by Dr. B. J. Tindall, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany, while the DNA:DNA hybridisations and determinations of G + C contents were done by Dr. R. Rosselló-Mora, Institut Mediterrani d`Éstudis Avançats, Mallorca, Spain.

CHAPTER 4. LC-MS analysis of samples of LADPEDS, and of its degradation products, was done in cooperation with Dr. M. J.-F. Suter and R. Schönenberger, Swiss Federal Institute for Environmental Science and Technology, Dübendorf, Switzerland. M. Lechner, University of Konstanz, helped on the growth experiments.

CHAPTER 5. LC-MS analyses of samples of LAS and SPC were done in cooperation with Prof. T. P. Knepper, Institute for Water Research and Water Technology, Wiesbaden, Germany. The synthesis of 3-(4-sulfophenyl)butyrate and the enrichment and isolation of *Comamonas testosteroni* KF-1 were done by K. Fischer, University of Konstanz.

CHAPTER 6. LC-MS analyses of samples of LAS and SPC were done in cooperation with Prof. T. P. Knepper (see above).

RESEARCH NOT INCLUDED IN THIS THESIS

In order to obtain a coherent thesis about my research done on the biodegradation of surfactants (see above), the results from other research were not included.

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CHAPTER 2

Parvibaculum lavamentivorans gen. nov., sp. nov., a new heterotrophic bacterium which initiates catabolism of linear alkylbenzenesulfonate (LAS)

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ABSTRACT

Strain DS-1^T is a small (0.8 μm in length and 0.2 μm in diameter) heterotrophic member of the α-Proteobacteria able to ω-oxygenate the commercial surfactant linear alkylbenzene-sulfonate (LAS) and shorten the sidechain by β-oxidation to yield sulfophenylcarboxylates. The morphotype is widespread in cultures able to utilise LAS, and a second organism with similar characteristics, strain AN-8, is now available. Utilisation of LAS is concomitant with formation of a biofilm, and cells are non-motile. Many surfactants were utilised. The organisms also grew with acetate or octane, but required no biofilm and were motile. Analysis of the gene encoding 16S rRNA placed the organisms in the α-subclass of Proteobacteria with a sequence divergence of > 8 % from any species whose name has been validly published. 16S rDNA sequence comparison to entries in the GenBank database showed 98 % similarity to an α-protobacterial marine isolate JP57: strain JP57 displayed the same morphotype as strain DS-1^T, but it was unable to utilise surfactants or any single source of carbon tested. The lipid components of strains DS-1^T and JP57 were virtually identical. The fatty acids contained ester- and putative amide-linked hydroxy fatty acids, in a combination which is currently unique in the α-Proteobacteria. The major respiratory quinone present in both strains was ubiquinone 11 (Q11) and the polar lipids consisted of phosphatidyl glycerol, diphosphatidyl glycerol, phosphatidyl ethanolamine, phosphatidyl choline and two unidentified aminolipids. Data on the 16S rRNA gene sequence and the lipid composition indicated that strains DS-1^T and JP57 should be placed in a new genus, for which we propose the name *Parvibaculum*. The differences between these strains, supported by DNA hybridisations, lead us to conclude that strain DS-1^T (= DSM 13023^T = NCIMB 13966^T) is the type strain of a species in the genus *Parvibaculum*, for which the name *Parvibaculum lavamentivorans* is proposed.

INTRODUCTION

Surfactants play an important part in our function and health, be they natural compounds in the lung or gut, naturally-derived compounds for clean skin (soap) or synthetic compounds for convenient cleaning (e.g. laundry). The major laundry surfactant is linear alkylbenzenesulfonate (LAS), whose world-wide production is about $2.5 * 10^6$ tonnes per annum (Schulze 1996). The complete biodegradation of LAS has been recognised for over 40 years (Sawyer and Ryckman 1957), but the first pure culture of a heterotrophic organism (strain DS-1^T) proven to utilise commercial LAS was not reported until 2000 (Schleheck *et al.* 2000; see also Hrsák and Begonja 1998). Strain DS-1^T catalyses the ω-oxygenation of the LAS sidechain and about three spirals of β-oxidation (Fig. 1); a wide range of products, sulfophenylcarboxylates (SPCs), sulfophenyldicarboxylates (SPdCs) and α,β-unsaturated SP(d)Cs are formed from commercial LAS, which nominally comprises 20 compounds (Schleheck *et al.* 2000; Dong *et al.* 2003, see also Eichhorn and Knepper 2002). Other organisms degrade the SPCs, SPdCs and α,β-unsaturated SP(d)Cs (Kanz *et al.* 1998; Schulz *et al.* 2000; Eichhorn and Knepper 2002; Schleheck *et al.* 2003c), so strain DS-1^T represents the first tier of the microbial community that degrades LAS.

Strain DS-1^T is an α-Proteobacterium (Schleheck *et al.* 2000). Other researchers have presumably failed to isolate it from enrichments, because it grows very slowly on complex medium (where it is rapidly overgrown by other organisms) and it is best separated on LAS-salts-agarose (where it can also be easily overgrown), and because it needs a solid support (e.g. glass fibre or polyester fleece) for growth with compounds such as LAS in liquid culture (Schleheck *et al.* 2000). We concluded that the organism represents the first of up to three tiers of bacteria needed to completely degrade commercial LAS (Schleheck *et al.* 2000; Schulz *et al.* 2000; Schleheck *et al.* 2003c) and we explored the degradation of LAS with six pristine and

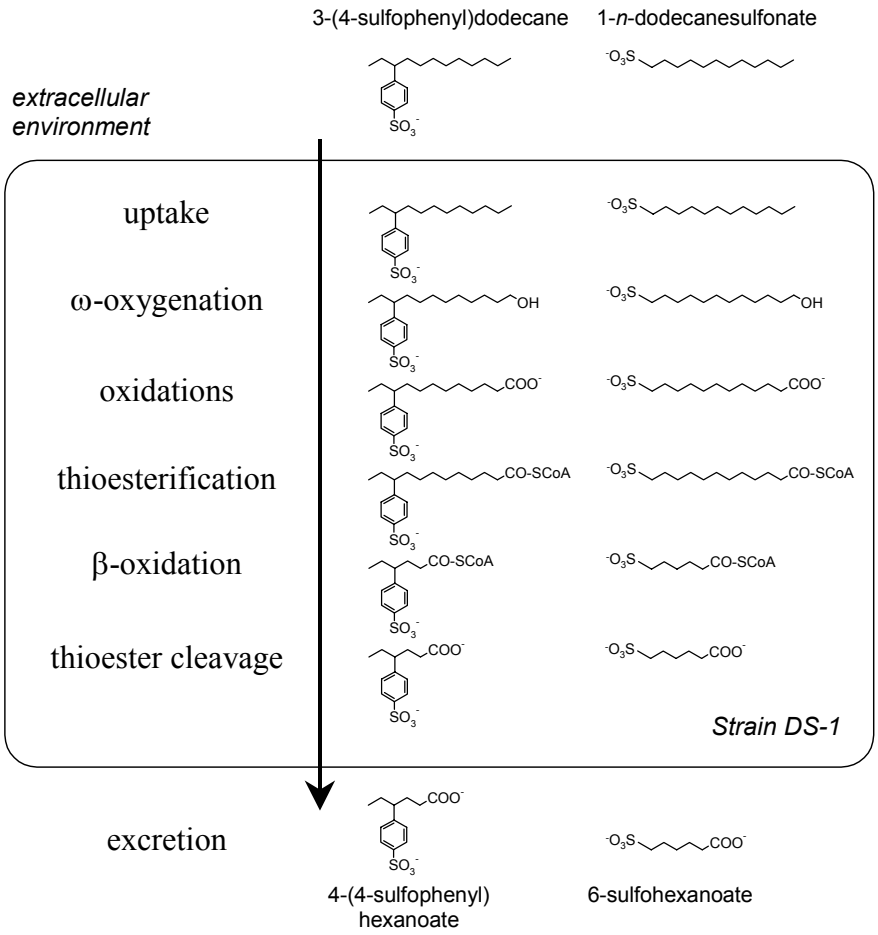


FIG. 1. Degradation of a representative congener of LAS by strain DS-1^T and the presumed degradation of a primary alkanesulfonate. Strain DS-1^T is apparently the first tier of different microbial communities (Schleheck *et al.* 2000; Schleheck *et al*. 2003c) which degrade many different surfactants (see Table 1).

acclimated inocula (Dong *et al.* 2003). This latter work supported the initial conclusion, with each culture containing organisms morphologically and physiologically similar to strain DS-1^T, and we were able to isolate one of those six organisms, strain AN-8 (Dong *et al.* 2003).

Strain DS-1^T was shown to be a member of the α-subclass of Proteobacteria (Schleheck *et al.* 2000) (as was strain AN-8), and represents a novel genus. Comparisons against other 16S rDNA sequences in databases indicated that marine α-Proteobacterium strain JP57 (Eilers *et al.*

2001) exhibited 98 % identity to strain DS-1, indicating that a related organism was available for comparison. Four uncultivated α-Proteobacteria, from freshwater and marine enrichment cultures which degrade hydrocarbons, also have similar rRNA genes (95-97 % identity to strain JP57) (Chang *et al.* 2000). We now confirm that strains DS-1^T and JP57 do not belong to the same species. We propose the name *Parvibaculum lavamentivorans*^{T} gen. nov., sp. nov., for strain DS-1^T; strain AN-8 is a second representative of *P. lavamentivorans*.

MATERIAL AND METHODS

Culture conditions

Strain DS-1^T or strain AN-8 was routinely grown in 3 ml of mineral-salts-medium (OECD 1992) in 30-ml screw-cap tubes to which up to 3 mM LAS (as carbon and energy source) and a solid support were added prior to autoclaving. Three solid supports were used, polyester fleece, an open-weave glass fibre and glass particles. Polyester fleece or glass fibre was cut to cubes (1 cm^3) and routinely added to 3-ml cultures (Schleheck *et al.* 2000); glass particles were generated by macerating glass fibre, and they were stored in stock suspensions (10 mg/ml) which were mixed to homogeneity with a magnetically-driven stirring bar and portions transferred in wide-mouthed pipette-tips to growth medium routinely to 1 mg/ml. [Caution: dry glass particles are a hazard to the eyes and lungs.] Cultures were inoculated (5 % v/v) with supernatant fluid from an outgrown LAS culture and incubated on a roller (100 rev/min) in the dark at 30 °C. The surfactants in routine use were commercial linear alkylbenzenesulfonate (C_{10}-C_{13} LAS) (Marlon A350; Hüls) and linear hexadecanediphenyletherdisulfonate (Dowfax 8390). The surfactants in Table 1 were tested as sole sources of carbon and energy for growth of strain DS-1^T. Each compound was provided at a concentration of 0.1 mM (calculated using the average chain length) in liquid minimal medium containing solid support and inoculated from an outgrown LAS culture (see above); very little growth was possible, but attack on the substrate could be estimated as loss of foaming in the culture (Schleheck *et al.* 2000). These cultures could be subcultured into medium containing 1 mM surfactant (and solid support) and growth (if any) was reproducible, as was loss of foaming as an indicator of degradation of surfactant. These cultures were also streaked on agar-salts medium which contained the appropriate surfactant. Growth with more usual (non-surface active) laboratory sources of carbon and energy did not need to be acclimated to the substrate to yield reproducible results. Volatile alkanes for plate cultures were provided in the gas phase. The identity of the organism subsequent to tests for substrate range was checked by plating on LB-medium, on plates with 1 mM-LAS-salts medium, and by behaviour in 1 mM-LAS-salts medium with and without solid phase. Pentane, octane, dodecane,

hexadecane or octanol (1 µl by syringe) was added directly to 3 ml liquid salts medium in a screw-capped tube. Hexadecanol or sodium hexadecanoate was provided as a suspension of particles in liquid culture medium.

Strain JP57, kindly provided by J. Peplies and R. Amann, MPI, Bremen, was routinely grown in a synthetic seawater-salts medium, MPM-m (Eilers *et al.* 2001), with peptone (10 g/L) as the carbon source, or in peptone medium (10 g Peptone, 5 g NaCl and 0.1 g [$CaCl_2$ x 2 H_2O] per L) in which strain DS-1^T also grew (cf. DSM medium 884). The substrate range of strain JP57 was tested in MPM-m medium, in MPM-m medium which was supplemented with 6 mM NH_4Cl and 0.25 mM K_2HPO_4, and in mineral-salts medium (see above), and to which peptone or a defined carbon source (see Table 1) was added. The used seven-vitamins solution (cf. DSM medium 503) and trace-elements solution (cf. DSM medium 320) are given elsewhere (Denger *et al.* 1999).

Morphology, physiology and biofilm staining

Culture purity, cell morphology, motility and spore formation were examined microscopically. The Gram reaction was assayed using the KOH test (Gregersen 1978). Oxidase and catalase tests were carried out following standard methods (Gerhardt *et al.* 1994). Strain DS-1^T grew in a biofilm on the polyester fleece when utilising LAS. The biofilm on a section of fleece from 0.5 mM LAS-salts medium was visualised *in situ* by staining with Ruthenium Red (2.5 µg/ml), whereas the cells were stained with DAPI (1 µg/ml).

Analytical methods

Respiratory lipoquinones and polar lipids were extracted from freeze dried cell material (100 mg) using a two stage method (Tindall 1990a; Tindall 1990b).

Respiratory lipoquinones were separated by TLC and UV absorbing bands corresponding to menaquinones or ubiquinones were removed from the plate and further analysed by reversed-phase HPLC. Polar lipids were separated by two dimensional silica gel TLC: total lipid material and specific functional groups were detected using dodecamolybdo-phosphoric acid (total lipids), Zinzadze reagent (phosphate), ninhydrin (free amino groups), periodate-Schiff (α-glycols), Dragendorff (quaternary nitrogen), and anisaldehyde-sulphuric acid (glycolipids) as described previously (Tindall 1990a; Tindall 1990b). Fatty acids were analysed as the methyl ester derivatives prepared from 10 mg of dry cell material. Cells were subjected to differential hydrolysis in order to detect ester linked and non-ester linked (amide bound) fatty acids (Tindall, unpublished). Fatty acid methyl esters were analysed by gas chromatography using a 0.2 µm x 25 m non-polar capillary column and flame ionisation detection. The run conditions were: injection and detector port temperature 300 °C, inlet pressure 60 kPa, split ratio 50:1, injection volume 1 µl, with a temperature program from 130 to 310 °C at a rate of 4 °C/min.

LAS and SPCs were determined by HPLC (Schleheck *et al.* 2000). Sulfate was quantified turbidimetrically (Sörbo 1987). Protein solublised from whole cells was quantified in Lowry-type reaction (Kennedy and Fewson 1968).

Extraction of genomic DNA, PCR-mediated amplification of the 16S rRNA gene, purification of the PCR products and sequence analysis were done as described elsewhere (Rainey *et al.* 1996). Sequence reactions were analysed using the Applied Biosystems 373A DNA Sequencer. The sequence was aligned manually to 16S rRNA gene sequences of representative microorganisms belonging to the domain *Bacteria* using the alignment editor ae2 (Maidak *et al.* 1996). A phylogenetic tree was generated using the algorithm of De Soete (De Soete 1983) (Carried out by the DSMZ, Braunschweig). DNA:DNA hybridisation was done as described previously (Ziemke *et al.* 1998). G + C-content was done as described elsewhere (Ziemke *et al.* 1998).

Accession numbers

The accession number of the 16S rDNA sequence of strain DS-1^T is AY387398. *P. lavamentivorans*^{T} DS-1 has been deposited with the German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany, under the accession number DSM 13023^T and with the National Collections of Industrial, Food and Marine Bacteria (NCIMB), Aberdeen, Scotland, UK under the accession number NCIMB 13966^T.

RESULTS AND DISCUSSION

Morphology and growth range

Strain DS-1^T was an aerobic, uniform, non-motile, short rod (0.8 µm in length and 0.2 µm in diameter), when growing with commercial LAS in suspended culture, associated with a biofilm (see also below) of the same organism on a solid support. The requirement for a solid support for growth with LAS is illustrated in Fig. 2AB. A non-inoculated culture foamed on shaking and turbidity due to glass particles was visible (Fig. 2A, left). The inoculum did not grow after 8 days, in the absence of particles, but in their presence strain DS-1^T degraded LAS (no foam on shaking) and caused the particles to clump due to the formation of biofilm (see sediment) (Fig. 2A, right). When a growth curve was generated (Fig. 2B), no growth was detected after 8 days without a support, but the addition of a small amount of glass particles (or polyester fleece, not shown) allowed growth; more glass particles allowed growth with a shorter lag phase. The organism did not grow in the presence of glass particles without LAS (not shown). Sorptive properties of the solid support were irrelevant for growth, since glass particles were non-sorptive, whereas polyester fleece could bind up to 50 % of the LAS (Fig. 2C), though long-chain congeners were more extensively bound. Strain DS-1^T showed no growth in the absence of glass particles (or fleece), as illustrated in Fig. 2AB, but prolonged incubation could lead to degradation of some LAS (not shown). The incubation time before degradation occurred could be extended by filtering the medium (0.2-µm pore size), so we presume that many types of particle can serve as a solid support for the development of a biofilm.

Strain DS-1^T was found to be able to metabolise a wide range of anionic and non-ionic surfactants (Table 1). In almost every case, a solid support was essential for growth. There were two apparent exceptions (Table 1), methylestersulfonate, which precipitated in the salts medium and thus supplied its own solid support, and octylbenzenesulfonate, for which we have no

TABLE 1. Growth of strain DS-1^T in liquid salts medium with various carbon sources and the effect of a solid support.

Carbon source and surfactant type [a] (if appropriate)		Growth	
		with solid support	without solid support
Linear hexadecanediphenyletherdisulfonate	A	+ [b]	-
Linear alkylbenzenesulfonate (sidechain $C_{10} - C_{13}$)	A	+ [b]	-
4-(1-*n*-Octyl)benzenesulfonate	A	+ [b]	+
4-Ethylbenzenesulfonate	-	-	
1-*n*-Dodecanesulfonate	A	+	-
sec-Alkanesulfonate ($C_{14} - C_{17}$)	A	+	-
α-Olefinsulfonate ($C_{14} - C_{16}$)	A	+	-
Methylestersulfonate ($C_{14} + C_{16}$)	A	+ [c]	+
Dodecane sulfate	A	+ [d]	-
Methyloleoyltaurate	A	+	- [e]
Methylcocoyltaurate	A	+	- [e]
Alkaneethoxylate sulfate	A	+	-
Lauryldiglycolether sulfate	A	+	- [e]
Cocoylglutamic acid	A	+	- [e]
Laurylsarcoside	A	+	-
Cholate	A	-	
Alkaneethoxylate	N	+	-
Tween 20, polyoxyethylene sorbitan monolaurate	N	+	-
Tween 80, polyoxyethylene sorbitan monooleate	N	+	-
Alkyl polyglucoside (C_8)	N	+	-
Fatty acid glucosamide (C_{10})	N	+	-

TABLE 1 continued

Triton X100, *iso*octylphenoxy polyethoxyethanol	N	-	
Brij-35, polyoxyethylene laurylether	N	-	
Brij-58, polyoxyethylene cetylether	N	-	
Cetyltrimethylammonium bromide	C	-	
Didecyldimethylammonium bromide	C	-	
Dimethyldecylamine	C	-	
Lauryldimethylbetaine	Z	-	
Cocoaminopropylsulfobetaine	Z	-	
Hexadecane	-	+[f]	+
Dodecane	-	+[f]	+
Octane	-	+[f]	+
Pentane	-	-	
Hexadecanoate	A	+[c,f]	+
Hexadecanol	-	+[c,f]	+
Ethanol	-	+[f]	+
Succinate	-	+[f]	+
Pyruvate	-	+[f]	+
Acetate	-	+[f]	+
Glycerol	-	-	
Glucose	-	-	
Fructose	-	-	
Galactose	-	-	

[a] Four types of surfactant were used: A, anionic; N, non-ionic; C, cationic; Z, zwitterionic.

[b] Substrate degradation and product formation were confirmed by HPLC analyses.

[c] Substrate formed a precipitate in minimal-salts medium.

[d] Ester sulfate recovered quantitatively as inorganic sulfate.

[e] Significant growth was independent of a solid support at surfactant concentrations <1 mM.

[f] The organism was motile under these conditions.

explanation. The only anionic surfactant (cholate), which did not support growth, contained no hydrocarbon chain. The non-ionic surfactants, which did not support growth, contain a branched hydrocarbon chain (Triton) or long polyethoxyethylene chains (Brij). Cationic surfactants were not utilised (Table 1), even when silica gel was added to reduce toxicity (see van Ginkel *et al.* 1992). Zwitterionic compounds were also not utilised (Table 1). Strain DS-1^T thus utilises all representatives of the two major classes of surfactants used in laundry products in Germany, anionics and non-ionics.

In contrast to the generality of needing a solid support for growth with surfactants, strain DS-1^T utilised some alkanes, alkanoates, alcohols and short chain acids (e.g. acetate), without the requirement for a solid support to the formation of a biofilm (Table 1). Under these latter conditions, the organism grew in suspension and was motile (Fig. 3A). Strain DS-1^T grew slowly in complex medium without a solid support, e.g. in peptone-salts medium (5 d): the organism was motile and was found as single cells or in short chains (2-5 organisms). The sugars tested were not utilised (Table 1).

Quantification of molar growth yields indicated that strain DS-1^T usually degraded alkyl chains of surfactants by excision of about six carbon atoms up to the hindrance of β-oxidation by respective (polar) substituents, as indicated in Fig. 1 for the utilisation of both LAS and dodecanesulfonate. No sulfate was recovered from any sulfonated surfactant.

Similarly, strain DS-1^T grew poorly with the alkylpolyglucoside and with the fatty acid glucosamide (Table 1), but did reduce the foaming, so presumably the short alkyl chain offered only about one spiral of β-oxidation for growth. In contrast, the growth yield with dodecyl sulfate indicated complete utilisation and, correspondingly, sulfate was recovered in high yield.

Growth of strain AN-8 with LAS also showed an absolute requirement for a solid support, concomitant with formation of a biofilm and the presence of non-motile cells. Growth with e.g. acetate or octane involved motile cells; no solid support was necessary and no biofilm

was formed. Growth in peptone-salts involved single cells and short chains. Sugars did not support growth. The cell morphology was indistinguishable from that of strain DS-1^T.

Marine isolate strain JP57 grew on synthetic-seawater agar plates without additional carbon source in pinpoint colonies, as described in the original publication (Eilers *et al.* 2001). The organism grew in peptone medium after 5 days of incubation and was morphologically indistinguishable from strains DS-1^T and AN-8. Strain JP57 failed to grow with LAS, sodium dodecyl sulfate, methylestersulfonate, octane, dodecane, hexadecane, hexadecanol, hexadecanoate, acetate, succinate, pyruvate, ethanol, or sugars as carbon source, when either supplied in minimal-salts medium, or when supplied in supplemented artificial sea-salts medium. We found no single source of carbon for the organism. Neither the addition of solid support nor a vitamin supplement had any effect.

Formation of biofilm

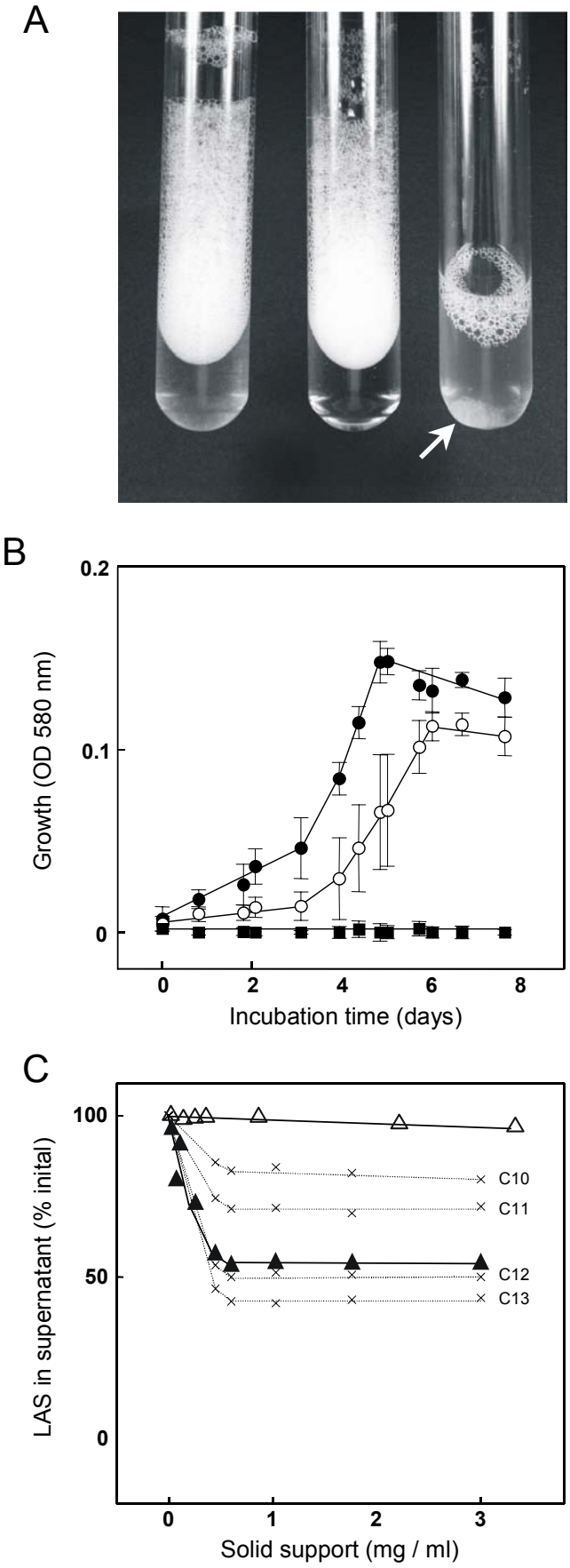
The first visible indication of growth of strain DS-1^T in LAS-salts medium was the formation of biofilm on the solid support; later in growth bacteria were also found in suspension. Two forms of biofilm could be observed on polyester fibres (Fig. 3BC). Form A was a densely packed sheath of cells around a fibre (Fig. 3B). Fig. 3B was generated with Ruthenium Red as the stain, but DAPI, Methylene Blue or Congo Red gave similar result (not shown). Form B was a gossamer (Fig. 3C) usually found linking two or more fibres. Each form could be removed from the support by e.g. vortexing and was of soft consistency. Form B only was observed on glass fibres (not shown), and negligible amounts of biofilm (form B only) was observed on glass particles, e.g. in the sediment from the culture fluid: the bacteria grew predominantly in suspension and were non motile.

We assume that the biofilm observed on glass fibres was continuously disrupted during incubation on a roller.

Growth in acetate-salts medium involved motile cells in suspension (see above). When LAS was added to an acetate-salts culture, a solid support was needed for growth; a biofilm was formed, and non-motile cells were observed in the presence of LAS.

Some growth substrates precipitated in the medium. In the case of methylestersulfonate surfactant (Table 1) a biofilm could be detected on the particles by staining (Ruthenium Red, not shown) and only non-motile cells were observed in the medium. In contrast growth with the insoluble substrates hexadecanoate or hexadecanol did not involve a biofilm (Ruthenium Red) and cells in the culture were motile. We presume that the formation of biofilm in strains DS-1^T and AN-8, and the switch from motile to non-motile, are protective responses to the risks inherent in membrane-solublising agents.

FIG . 2 (opposite page)**. Growth of strain DS-1^T in LAS-salts medium as a function of the presence of solid support assayed as the disappearance of substrate (A) or as growth (B) and the sorbtion of LAS to different supports (C).** Cultures of strain DS-1^T were incubated for 8 days (A, B). The spot test (shaking) indicated the presence of surfactant as foam (A): non-inoculated control with glass particles (1 mg/ml) (left); inoculated culture in absence of glass particles (centre); inoculated culture in the presence of glass particles (1 mg/ml) (right), where the arrow indicates the sediment of clumped glass particles. Growth could be assayed as turbidity (OD 580 nm) after shaking to detach the biofilm from the support, and allowing the glass particles to settle (B). The values are the mean of three independent experiments and the error bars show the standard deviation: (■) no glass particles; (○), 0.5 mg glass particles per ml; (●), 1 mg glass particles per ml. Surfactants can be difficult to quantify in growth medium, which is illustrated in C, where a fixed concentration of LAS in sterile salts medium was incubated with different amounts of solid support (polyester fibre or glass particles), and the LAS concentration in solution was determined after 12 h (HPLC). Values of the negative controls (no solid support) were set as 100 %. LAS concentration in solution (solid lines) in the presence of polyester fleece (▲) or glass fibre (Δ). Detailed analysis of HPLC chromatograms showed that a higher proportion of the longer-chain congeners of LAS sorbed to the fleece, whereas there was no sorption to the glass particles. LAS desorbs from the fleece during growth: the same concentration of SPC is observed at the end of growth in the presence of glass particles or fleece (not shown).


A
B
0.2
0.1
0
Growth (OD 580 nm)
0
2
4
6
8
Incubation time (days)
C
100
50
0
LAS in supernatant (% inital)
C10
C11
C12
C13
0
1
2
3
Solid support (mg / ml)

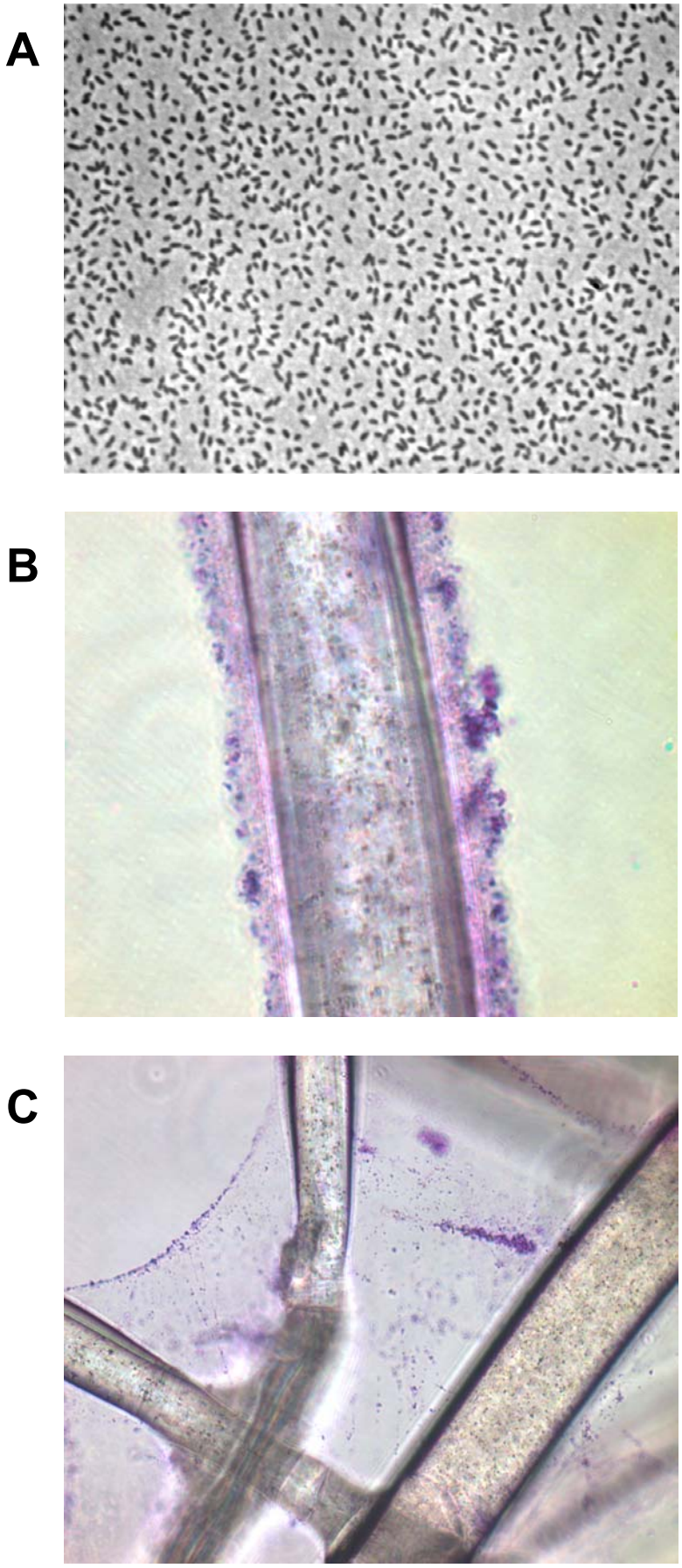


FIG. 3. Photomicrographs of phase-contrast microscopy of strain DS-1^T during growth in acetate-salts medium (A) or during growth in LAS-salts medium as biofilm on polyester fleece (B, C) (stain, Ruthenium Red). Each scale bar represents 10 μm.

16S rDNA sequence analysis, chemotaxonomy and DNA:DNA hybridisation

Published data on the sequences of 16S-rRNA genes place strains DS-1^T (1451 nt) and JP57 (1356 nt), which share 98 % similarity, in the α-Proteobacteria, where the nearest, well-described organism is *Rhodobium* (formerly *Rhodobacter*) *marinum*, which shares only 92 % similarity (Schleheck *et al.* 2000; Eilers *et al.* 2001). The morphological similarities of these two strains supports the implication in the sequence that they are closely related, but differences are also apparent. A 400 bp sequence of 16S-rRNA gene from strain AN-8 was identical with the corresponding sequence of strain DS-1^T, which, with the essentially identical morphology and physiology, supports the hypothesis that they are members of one species.

Examination of the respiratory lipoquinone composition of both strain JP57 and DS-1^T showed that ubiquinones were the sole respiratory quinones present. Furthermore, the major lipoquinone had eleven isoprenologues in the side chain, i.e. ubiquinone 11 (Q11).

The fatty acids comprised both saturated and unsaturated straight chain fatty acids, as well as hydroxylated fatty acids, both strains showing only quantitative differences (Table 2). Differential hydrolysis of the cells indicated that some of the hydroxyl fatty acids were probably amide linked (Table 2). The polar lipids comprised the phospholipids, phosphatidyl glycerol, diphosphatidyl glycerol, phosphatidyl ethanolamine, and phosphatidyl choline (Fig. 4). In addition two aminolipids were present which ran between phosphatidyl glycerol and phosphatidyl choline (Fig. 4). There were quantitative differences in the ratio of these two lipids, with one predominating in strain JP57. This causes the lower lipid to be partially obscured by the upper lipid, but this was simply a `crowding effect´ on the TLC plate.

The G + C content of strain DS-1^T and JP57 were determined to be 64.5 (± 1) % and 63.7 (± 1.3) %, respectively.

TABLE 2. Percentage composition of the fatty acids present in strain DS-1^T and JP57.

Fatty acid	Strain DS-1^T		Strain JP57	
	Method 1 [a]	Method 2 [a]	Method 1 [a]	Method 2 [a]
3-OH 14:0	6.3	5.7	5.3	5.5
16:0	6.5	6.1	2.7	2.4
3-OH 16:1	1.4	1.3	tr	tr
3-OH 16:0 [b]	1.2	9.1	tr	7.6
18:1ω7c	50.4	47.4	72.1	66.4
18:0	9.7	9.0	3.4	3.2
unknown	tr	tr	0.9	1.2
cyclo 19:0	13.6	11.8	6.7	5.3
2-OH 18:1	2.8	2.6	2.5	2.5
cyclo 2-OH 19:0	8.1	7.0	6.4	5.9

[a] Methods 1 and 2 released ester-linked and ester-linked plus amide-linked fatty acids, respectively.

[b] Putative amide-linked fatty acids.

tr Trace amounts.

The use of a combination of the 16S rRNA sequence data and the lipid composition provides a convenient way of quickly determining the differences and/or similarities to other organisms. The presence of ubiquinones as the sole respiratory quinones is indicative of the fact that strains DS-1^T and JP57 are members of the α-, β-, or γ-subclass of the Proteobacteria. The presence of ubiquinone 11 is distinctive in that the presence of this compound as the sole major quinone has only previously been found in members of the genus *Hyphomonas* (Urakami and Komagata 1987; Sittig and Hirsch 1992). Ubiquinone 11 is also known to occur in members of the genus *Legionella*, (a member of the γ-subclass of the Proteobacteria) but it is usually accompanied by additional isoprenologues (see Collins and Gilbart 1983).

Fatty acid patterns of the two strains, showing large amounts of 18:1ω7c is not atypical of members of the α-subclass of the Proteobacteria. However, the distribution of the hydroxy fatty acids does appear to be a unique feature of these two organisms. In particular, the fact that some of the hydroxyl fatty acids are ester linked, while others are amide linked, allows further differentiation of these two strains from other members of the α-subclass of the Proteobacteria. The presence of the phospholipids phosphatidyl glycerol, diphosphatidyl glycerol, phosphatidyl ethanolamine, and phosphatidyl choline is also characteristic, together with the respiratory lipoquinone and fatty acid data, of certain subgroups within the α-subclass of the Proteobacteria. However, the presence of the two additional aminolipids appears to be distinctive of these two organisms.

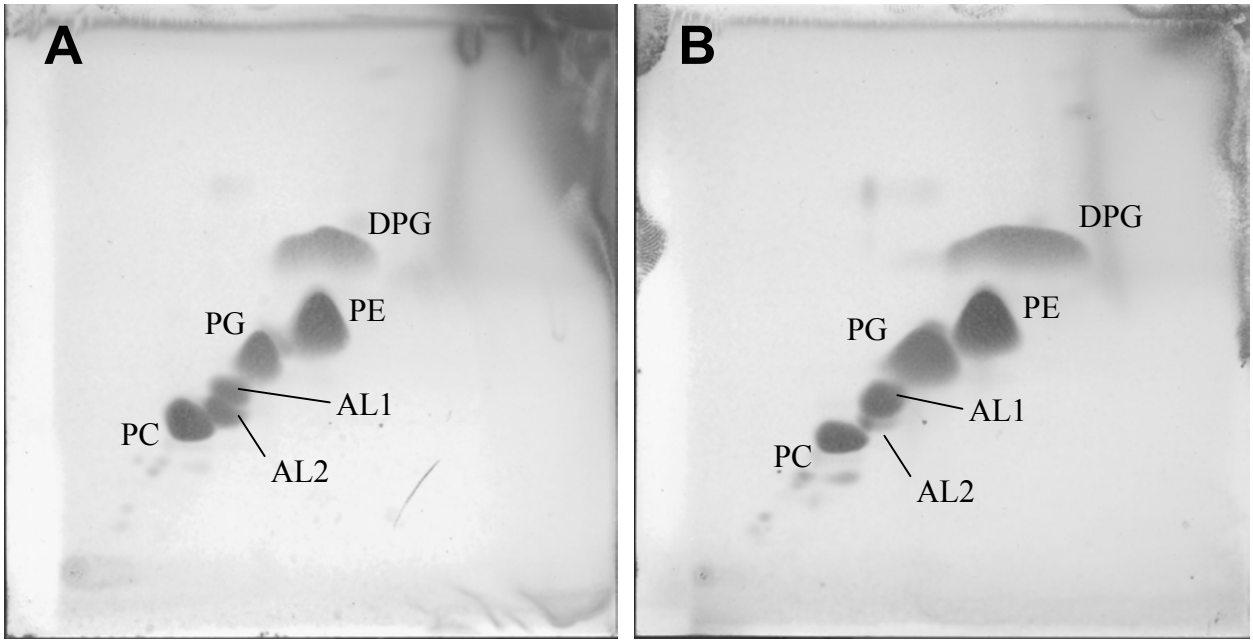


FIG. 4. Two-dimensional thin layer chromatogram of the polar lipids of strain DS-1^T (A) and JP57 (B) stained with 5% ethanolic molybdo-phosphoric acid. Solvents: first dimension, chloroform:methanol:water (65:25:4 v/v/v); second dimension, chloroform:methanol:acetic acid:water (80:12:15:4 v/v/v). DPG, diphosphatidyl glycerol; PE, phosphatidyl ethanolamine; PG, phosphatidyl glycerol; PC

DNA:DNA hybridisation with strains DS-1^T and JP57 showed 42 % binding, similar to the value obtained against a strain of *Pseudomonas putida* (35 %), whereas the positive control was 100 %. We thus conclude that strains DS-1^T and JP57 represent different strains of the same higher taxon within the α-subgroup of Proteobacteria. In the present work we have concentrated on the taxonomic position of strain DS-1, and a taxonomic treatment of strain JP57 may appear at a later date.

Description of *Parvibaculum lavamentivorans* gen. nov., sp. nov.

Par.vi.ba'cu.lum. L. adj. *parvus* small; L. neut. n. *baculum* stick; L. neut. n. *parvibaculum* small stick.

la.va.men.ti.vo'rans. L. v. *lavo* to wash, L. neut. n. suffix *-mentum* agent of (specified) action; L. v. *voro* to consume; L. neut. part. adj. *lavamentivorans* consuming (chemicals) used for washing.

Aerobic, uniform, short rod (0.8 μm in length and 0.2 μm in diameter). Oxidase and catalase positive. Mesophilic. Motile when growing with acetate, octane, or in complex medium.

Ubiquinone 11 (Q11) as major respiratory quinone. Straight chain saturated and unsaturated, as well as ester- and amide-linked hydroxy-fatty acids, in membrane fractions (see Table 2). The major polar lipids are phosphatidyl glycerol, diphosphatidyl glycerol, phosphatidyl ethanolamine, phosphatidyl choline, and two, unidentified aminolipids. 16S rDNA sequence analysis indicates this taxon as member of the α-subclass of the Proteobacteria, with > 8 % sequence divergence to any other species within this subclass, whose name has been validly published. The G + C content is 64 %.

Strain DS-1 grows with linear alkylbenzenesulfonate (LAS) surfactant and other surfactants *via* ω-oxygenation and β-oxidation of the alkyl chain to produce short-chain carboxylates. Growth in surfactant medium involves biofilm formation on solid support (polyester fleece, glass particles) and non-motile cells. The type species of the genus is *Parvibaculum lavamentivorans.* Strain DS-1 is the type strain of *Parvibaculum lavamentivorans*,

and is deposited with the DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH under DSM 13023^T and with the National Collection of Industrial and Marine Bacteria under NCIMB 13966^T.

ACKNOWLEDGEMENTS

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CHAPTER 3

ω-Oxygenation of linear alkylbenzenesulfonate (LAS) by a cytochrome P450 monooxygenase in *Parvibaculum lavamentivorans*^{T} DS-1

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Archives of Microbiology,
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ABSTRACT

Heterotrophic *Parvibaculum lavamentivorans*^{T} DS-1 degrades linear alkylbenzene-sulfonate (LAS) surfactant during growth to yield sulfophenylcarboxylates (SPC) of shortened side-chain length via ω-oxygenation and β-oxidation. The LAS-degradative enzymes were apparently active also during growth of strain DS-1^T with acetate: when LAS was added in a growth experiment to an acetate-growing culture, the immediate conversion of LAS into SPC in an identical pattern was observed, and lysis of part of the growing culture. In a experiment where LAS and acetate were both present, growth involved the complete utilisation of LAS with formation of SPC, before acetate was utilised. Specific LAS-dependent oxygen uptake was detected in LAS- and in acetate-grown whole cells with a maximal activity at about 1.0 or 0.6 mkat/kg protein shortly before the end of growth with LAS or acetate, respectively, but not in freshly prepared extracts of whole cells; activity was also detected when a wide range of anionic and nonionic surfactants, or octane, was tested. Whole cells did not convert LAS to SPC in the absence of molecular oxygen, and not in presence of molecular oxygen when pretreated with the cytochrome P450-specific inhibitor metyrapone. The oxygen uptake in whole cells was inhibited by metyrapone in an concentration-dependent manner. Absorption maxima indicative of a cytochrome P450-like protein present in the soluble fraction of crude extract were observed when difference spectra of oxic *versus* reduced extracts, or of carbon-monoxide saturated reduced extract *versus* reduced extract, were taken. We infer that a soluble, multi-component, P450-dependent monooxygenase system catalyses ω-oxygenation of LAS, and that the complete LAS-degradative enzymes are constitutively expressed in strain DS-1^T.

INTRODUCTION

There is one widespread pathway to degrade commercial linear alkylbenzenesulfonate (LAS) surfactant. This involves a complex microbial community (Dong *et al.* 2003; Schleheck *et al.* 2003c), and a complex mixture of short-chain sulfophenylcarboxylates (SPC) and sulfophenyldicarboxylates (SPdC) as transient intermediates (Eichhorn and Knepper 2002; Dong *et al.* 2003; Schleheck *et al.* 2003b; Schleheck *et al.* 2003c) (in the following denoted as SP(d)C).

Parvibaculum lavamentivorans^{T} DS-1 is a representative environmental isolate that catalyses the initial degradation of LAS to SP(d)C during heterotrophic growth (Schleheck *et al.* 2000; Dong *et al.* 2003; Schleheck *et al.* 2003e). Growth of the organism in the laboratory with LAS in liquid culture involves the disappearance of foam, the dependence on the presence of solid support in the culture fluid (e.g. glass particles), and the formation of a biofilm in the early growth phase (Schleheck *et al.* 2003e). The organism utilises a wide range of anionic and nonionic surfactants for growth (Schleheck *et al.* 2003e).

The inferred degradation pathway of LAS in strain DS-1^{T} (Dong *et al.* 2003; Schleheck *et al.* 2003c), and presumably of the other surfactants utilised, is principally similar to the degradation pathway for utilisation of *n*-alkanes, and strain DS-1^{T} is able to utilise medium-chain alkanes for growth (C_8-C_{16}; Schleheck *et al.* 2003e). The activity of a presumed multi-component ω-oxygenase system was detected in strain DS-1^{T} (Schleheck *et al.* 2000), thus to introduce a terminal hydroxyl group at the alkyl chain of LAS (C_{10}-C_{13}), and to yield long-chain sulfophenylcarboxylates (lc-SPC, C_{10}-C_{13}) after two following oxidations catalysed by separate enzymes (Fig. 1). The lc-SPCs then undergo chain-shortening through the sequential excision of acetyl-CoA (β-oxidation), which leads to mainly C_4-C_9 SPC when further β-oxidation is apparently hindered by the sulfophenyl substituent, and are excreted (Eichhorn and Knepper

2002; Dong *et al.* 2003; Schleheck *et al.* 2003c); an extension of this concept involves a second ω-oxygenation and sulfophenyldicarboxylates (SPdC) (Dong *et al.* 2003; Schleheck *et al.* 2003b).

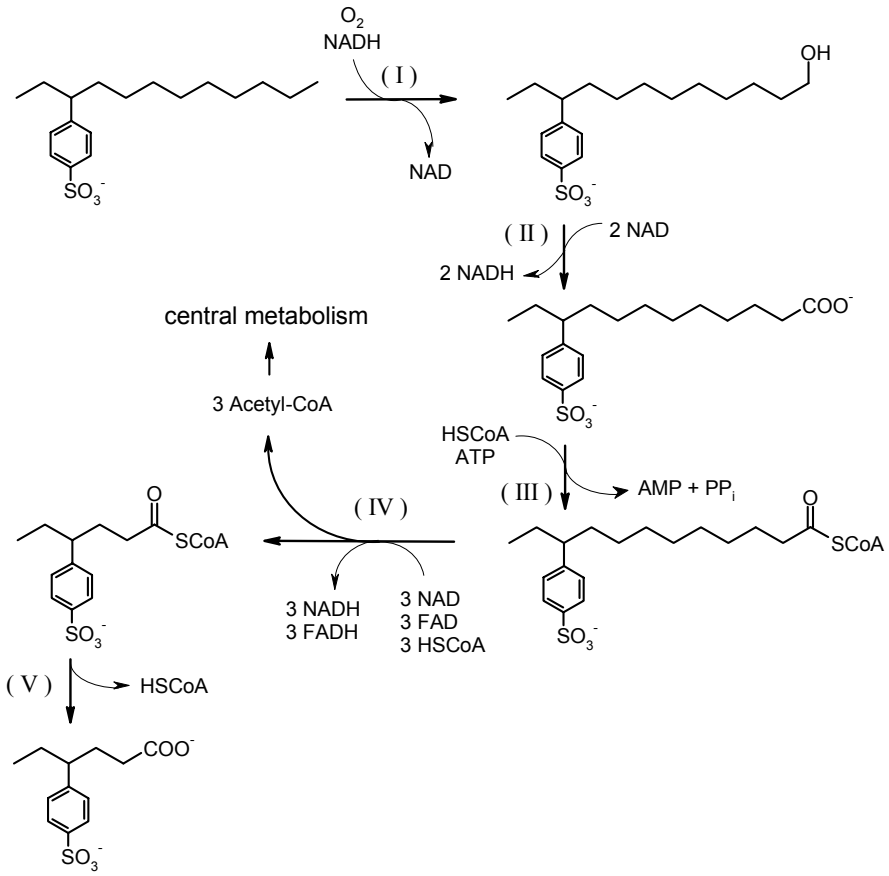

FIG. 1 LAS-degrading reactions in *P. lavamentivorans*^{T} DS-1. The illustration shows 3-(4-sulfophenyl)dodecane (3-C12-LAS) as a representative LAS congener, which is degraded into 4-(4-sulfophenyl)hexanoate (4-C6-SPC), and excreted. Reactions catalyzed after uptake of LAS: (I), ω-oxygenation; (II), ω-oxidations; (III), thioesterification; (IV), spirals of β-oxidation contributed by α,β-desaturation, addition of water, oxidation, and β-ketothiolysis; (V) thioester cleavage.

The ω-oxygenation of *n*-alkanes is catalysed in many Gram-negative and Gram-positive bacteria by the well-known Alk-type hydroxylase systems (Smits *et al.* 1999; Smits *et al.* 2002; van Beilen *et al.* 2002), e.g. in *Pseudomonas oleovorans* GPo1. The system is constituted by alkane hydroxylase (AlkB), by rubredoxin (AlkG), and by rubredoxin reductase (AlkT); AlkB is a non-heme di-iron integral membrane protein which carries out the ω-oxygenation (McKenna and Coon 1970; Kok *et al.* 1989; van Beilen *et al.* 1992), and rubredoxin transfers electrons from the NADH-dependent flavoprotein rubredoxin reductase to AlkB (Peterson *et al.* 1966; Ueda *et al.* 1972).

Furthermore, cytochrome P450-heme dependent monooxygenases are known to catalyse ω-oxygenation of *n*-alkanes in bacteria, the Non-system of *Acinetobacter* sp. EB104 (Müller *et al.* 1989; Maier *et al.* 2001), which is constituted by *n*-alkane monooxygenase (CYP153 protein, NonM), ferredoxin (NonF) and ferredoxin reductase (NonO), or derivatives of the long-chain fatty acid ω-2 (subterminal) monooxygenase $P450_{BM-3}$ of *Bacillus megaterium,* a catalytically self-sufficient protein containing P450-heme and electron-transfer domains (Narhi and Fulco 1986; Ravichandran *et al.* 1993), after laboratory-evolution (Glieder *et al.* 2002; see also Oliver *et al.* 1997). P450-type *n*-alkane ω-oxygenases are found widely spread in eukaryotic organisms, e.g. in alkane-degrading yeast (e.g. Scheller *et al.* 1996; Iida *et al.* 2000).

We further characterised the physiology of growth of the of strain DS-1^T, the LAS-degradative pathway, and the ω-oxygenase activity as displayed by whole cells. The results indicate that the LAS-degradative pathway is constitutively expressed in strain DS-1^T, and that the ω-oxygenase enzyme is apparently constituted by a cytochrome P450-heme dependent, multi-component oxygenase system.

MATERIALS AND METHODS

Growth media and growth conditions

P. lavamentivorans^T DS-1 (DSM 13023^T, NCIMB 13966^T) was grown in culture tubes on a roller, or in Erlenmeyer flasks on a orbital shaker, as described previously (Schleheck *et al.* 2000; Schleheck *et al.* 2003e), or in a 3.5-L fermenter at 30 °C in the dark. A mineral salts medium was used, and glass particles (1 mg/ml final) were added to the culture fluid when the organism was grown with LAS (Schleheck *et al.* 2003e). Glass particles sedimented from the culture fluid, and cells in the supernatant could be collected by centrifugation.

Growth experiments were done in a fermenter (Schütt, Göttingen, Germany). Samples were taken at intervals to determine the concentrations of substrate and products, to measure turbidity and assay the protein concentration, and to measure the specific oxygen uptake rates of the culture. The cultures were aerated through injection of filter-sterilised air into the culture fluid during growth with acetate. During growth with LAS, the fermenter was operated with the following modifications to prevent excessive foaming of the surfactant: the medium was stirred by propellers directing the liquid in the center downwards, so that the foam from the surface was submerged into the culture fluid as stream of bubbles; the culture was aerated through a glass pipe, which delivered filter-sterilised air onto the surface of the culture fluid, and was pointed onto the vortex.

Experiments with whole cells

Cells were harvested by centrifugation (8,000 g, 15 min, 4 °C), washed twice and resuspended in chilled 50 mM potassium phosphate buffer, pH 7.2, and immediately used to assay the substrate-dependent oxygen uptake at 30 °C in a Clarke-type electrode (0.5 ml, 1.7 – 2.8 mg protein/ml). The substrates were added routinely to give 80 µM final concentration, and after the reactions, the substrate turnover and product formation was assay by HPLC-analysis. The effect of monooxygenase-inhibitors (see Results) was tested when the cells were pretreated with the inhibitor. Substrate degradation and product formation was followed by HPLC in up-scaled reactions mixtures (6 ml) aerated by magnetically stirring at 30 °C. The reactions were started by substrate addition (80 µM), and samples (0.1 ml) were taken at intervals and added to 0.9 ml 0.05 M phosphoric acid, and then centrifuged. The supernatant fluid was analysed by HPLC.

Atmospheric oxygen was removed from 6-ml cell suspensions in 15 ml septum vials by degassing under vacuum and purging with nitrogen gas through needles in the septum. The reaction was started by addition of substrate (80 µM), and samples were taken at intervals through the septum for HPLC analysis.

Experiments with crude extract

Cells from which crude extract was to be prepared were grown in 30 mM acetate-salts medium, and harvested in the late-exponential growth phase ($OD_{580\ nm}$ 0.8 - 1.0) when the specific activity of the oxygenase enzyme was optimal; the washed cells were used immediately, or stored frozen at -20 °C. Cells were resuspended in three or four volumes of 50 mM Tris-HCl buffer, pH 7.8, which contained 10 % (v/v) glycerol, 2 mM $MgSO_4$ and 0.1 mg/ml DNase, and disrupted by passages through a French pressure cell (Schleheck and Cook 2003a). Whole cells and debris were removed by centrifugation (14,000 g, 15 min, 4 °C). The membrane fraction was removed by ultra-centrifugation (50,000 g, 30 min, 4 °C).

We assayed the substrate-dependent oxygen uptake in crude extract and in membrane-free crude extract in 50 mM Tris-HCl buffer, pH 7.8. The substrates were present at 80 µM. The net reaction was not altered in the presence of 1 mM NADH or NADPH. Substrate degradation and product formation was determined by HPLC after the reactions, or was followed at intervals in scaled-up reactions mixtures (3 ml) aerated by magnetically stirring at 30 °C.

Difference spectra of crude extract were taken photometrically between a sample with oxic crude extract *versus* a reference sample with crude extract treated with dithionite (2 mM final), and between a sample of carbon-monoxide saturated dithionite-treated crude extract *versus* a reference sample with dithionite-treated crude extract. For carbon-monoxide saturation, dithionite-treated crude extract in 5 ml septum vials was degassed under vacuum, purged with carbon-monoxide gas through needles in the septum, and analysed after 10 min. The membrane fraction obtained after ultracentrifugation of 3 ml oxic crude extract was solublised in 3 ml Tris-HCl buffer, pH 7.8, which contained 0.5 % SDS and 10 % (v/v) glycerol, and 1.5 ml therefrom were treated with dithionite (2 mM) to obtain the reference sample.

Analytical methods

Growth was followed as turbidity and quantified as protein in a Lowry-type reaction (Kennedy and Fewson 1968) (OD_{580} = 1.0 = 310 mg protein/L). Protein in crude extracts and in supernatant fluid of culture medium after centrifugation was assayed by protein-dye binding (Bradford 1976). DNA in supernatant fluid of culture medium after centrifugation was assayed fluorometrically, using the DNA-specific stain Hoechst H33258 and an DyNA Quant apparatus (Hoefer-Pharmacia).

LAS and SPC was determined by HPLC with the gradient system as described previously (Schleheck *et al.* 2000) (0.11 M $NaClO_4$ and acetonitrile as mobile phases), where the peaks of the major SPCs could be identified on the chromatograms following the interpretations of data obtained in earlier work (see Dong *et al.* 2003; Schleheck *et al.* 2003b; Schleheck *et al.* 2003c). Acetate was determined by gas-chromatography as described elsewhere (Laue *et al.* 1997).

Polymerase chain reactions

PCR reactions were carried out in a Mastercycler-Gradient apparatus (Eppendorf) (Schleheck 2000) using the conditions indicated in the original publication for the amplification of a fragment of *alkB*-related alkane hydroxylase genes (Smits *et al.* 1999, primer-pair TS2S - deg1RE). Chromosomal DNA from strain DS-1^T was isolated according to Ausubel et *al.* (Ausubel *et al.* 1987).

Chemicals

Metyrapone (1,2-Di-[3-pyridyl]-2-methyl-1-propanone) and Methimazole (2-Mercapto-1-methylimidazole) were purchased from Aldrich (Steinheim, Germany). The sources of surfactants and of the routine chemicals used are stated elsewhere (Schleheck *et al.* 2000; Schleheck *et al.* 2003b; Schleheck *et al.* 2003c).

RESULTS AND DISCUSSION

Physiology of growth of *P. lavamentivorans*^{T} DS-1

Strain DS-1^T grew in 1 mM LAS-salts medium only in the presence of a solid support (glass particles), and LAS was converted into SP(d)C (Dong *et al.* 2003; Schleheck *et al.* 2003b; Schleheck *et al.* 2003c). The growth yield (43 µg protein/ml) indicated about 6.5 carbon atoms utilised from commercial LAS in average (Schleheck *et al.* 2003e), and the growth involved clumping of the glass particles due to formation of some biofilm, and mostly suspended, non-motile organisms when observed in the late-exponential growth phase, and stationary phase (Schleheck *et al.* 2003e).

The organism grew with a specific growth rate of $\mu = 0.028\ h^{-1}$ in culture tubes, and a specific degradation rate of LAS of 0.18 mkat/kg protein could thus be calculated from the specific growth rate in culture tubes, and the growth yield we measured. The organism grew with lower rate in larger culture volume in Erlenmeyer flasks ($\mu = 0.018\ h^{-1}$, 1 L), or under optimised conditions in a fermenter ($\mu = 0.022\ h^{-1}$, 3.5 L) (Fig. 2A). Large amounts of foam covered the liquid in Erlenmeyer flasks (orbital shaked) and in the fermenter (stirred), but not in culture tubes (rolled), and we presume that the lower growth rates resulted from limited aeration.

The activity of ω-oxygenase (see below) could be detected in whole cells from cultures growing with 1 mM LAS when analysed in the oxygen electrode after addition of LAS. The cells exhibited substrate-dependent oxygen uptake, and the concomitant conversion of LAS into SPC was confirmed by HPLC analysis (see below). During growth with LAS (Fig. 2A), the activity steadily rose to a maximum of about 1.1 mkat/kg protein shortly before the end of growth, and fell to 0.5 mkat/kg protein two days after the culture was outgrown. The pattern of specific activity thus confirmed our earlier observations (Schleheck *et al.* 2000), indicating a behaviour typical of many multi-component oxygenase systems (e.g. Junker *et al.* 1994a; Junker *et al.*

1994b; Schleheck and Cook 2003a); the effect is apparently due to the specific degradation of one component of the oxygenase system (Moodie *et al.* 1990). We thus presume that the ω-oxygenation of LAS is catalysed by a multi-component oxygenase system in strain DS-1^T.

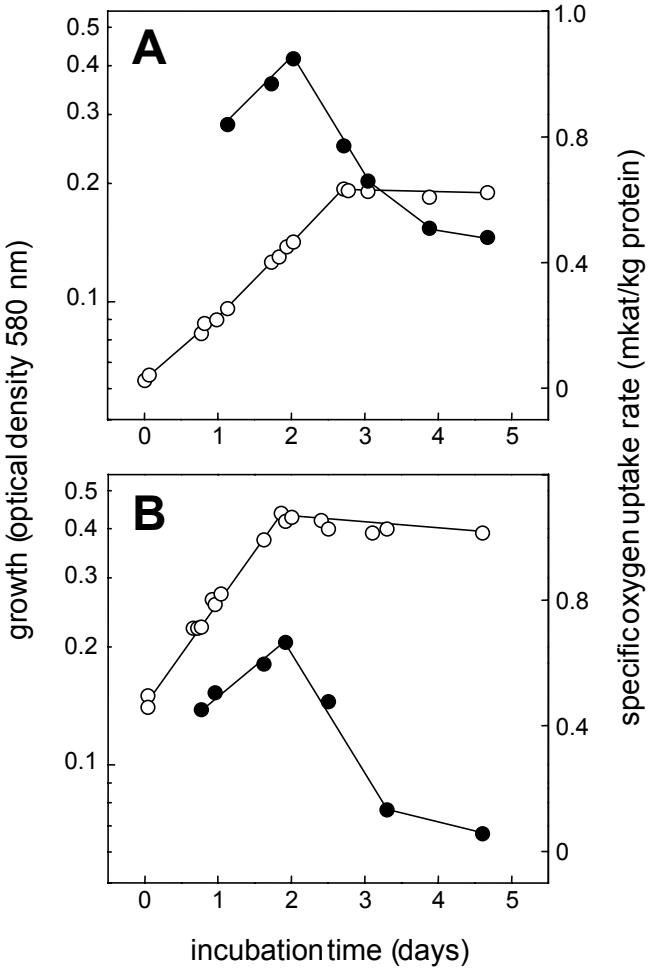

FIG. 2. Growth of strain DS-1^T in 1 mM LAS salts medium (A) and 10 mM acetate-salts medium (B) and the specific LAS-dependent oxygen uptake rates of whole cells. The batch-cultures were grown in a fermenter (see Methods). Symbols: ❍, optical density; ●, specific oxygen uptake rate.

Strain DS-1^T grew in acetate-salts medium independent of the addition of glass particles as motile organism, as observed previously (Schleheck *et al.* 2003e), with a specific growth rate of 0.039 h^{-1} (Fig. 2B) independent of the culture vessel used. The molar growth yield obtained was 6.6 g protein/mol carbon, a normal value (Cook 1987). Acetate-growing cells also exhibited LAS-dependent oxygen uptake when tested, though with lower specific activity (Fig. 2B). The activity rose steadily to a maximum of about 0.6 mkat/kg protein shortly before the end of growth, and fell to 0.1 mkat/kg protein within about two days, a behaviour similar to that observed during growth with LAS. Furthermore, LAS was degraded to SP(d)C (see below), and we presumed that the enzymes of the LAS-degradative pathway were active during growth with acetate.

We could confirm this in an experiment, in which LAS was added to an acetate-growing culture (Fig. 3). Growth continued after the addition, though the specific growth rate dropped from $\mu = 0.041\ h^{-1}$ before addition to $\mu = 0.024\ h^{-1}$ after addition of LAS (Fig. 3A), and lysis of part of the culture after addition of the surfactant was indicated by the release of protein and DNA into the culture medium (Fig. 3A).

The immediate formation of SPC was detected after addition of LAS (Fig. 3B), with the concomitant disappearance of LAS; the fate of LAS during the first 15 min after addition was attributed to sorption of the surfactant to the biomass (about 13 %). A specific degradation rate of LAS of about 0.05 mkat/kg protein could be calculated from the rate of LAS disappearance and the protein concentration.

In this experiment, LAS was subject to conversion into SP(d)Cs in an identical pattern when compared to the conversion during growth of strain DS-1^T with LAS (cf. Schleheck *et al.* 2003c), as illustrated in Fig. 4: the 2- and 3-substituted LAS congeners disappeared first, and short-chain SPCs were excreted (Fig. 4AB, see also the inset). The centrally-substituted LAS congeners were degraded later and more slowly, and yielded medium-chain SPCs (Fig. 4CD, inset). This behaviour represented the `distance principle´ of degradation of LAS as observed

repeatedly (Swisher 1987; Schöberl 1989), in that LAS congeners with longer, linear alkyl-side chain are degraded faster than the congeners with short alkyl-side chain. This experimental setup yielded for the first time long-chain SPCs as transient intermediates (Fig. 4CD).

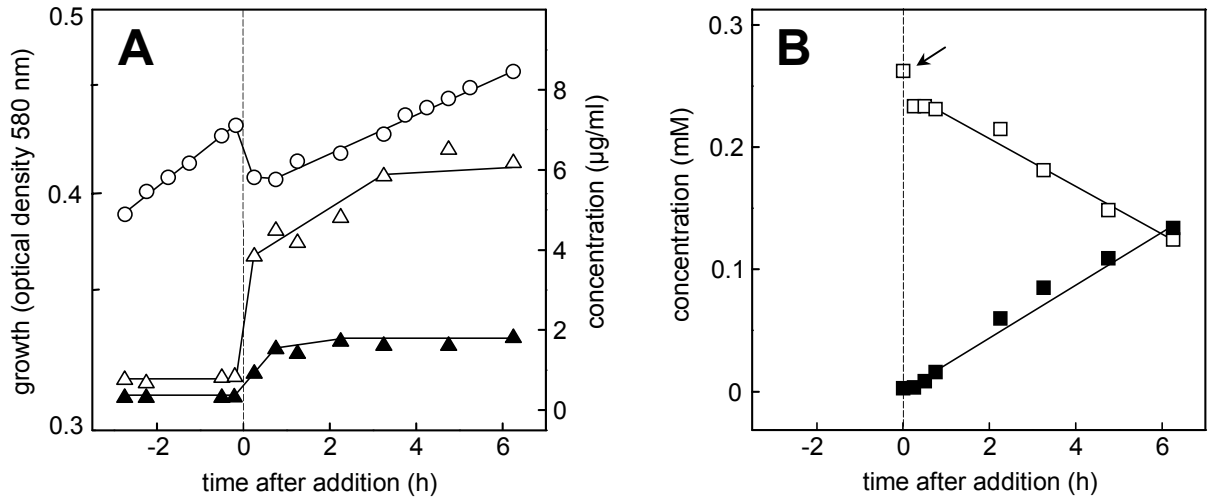

FIG. 3. Growth of strain DS-1^T in acetate-salts medium and the concentration of protein and DNA determined in the culture fluid after addition of LAS (A), and the concentration of LAS and SPC (B). LAS-solution was added to a 500-ml culture to give a final concentration of 0.25 mM. The protein and DNA concentration was determined in the supernatant fluid of samples after centrifugation (see Methods). The initial concentration of LAS was determined in a control where no biomass was present (arrow). Symbols: ❍, optical density; Δ, protein; ▲, DNA; □, total LAS; ■, total SP(d)C.

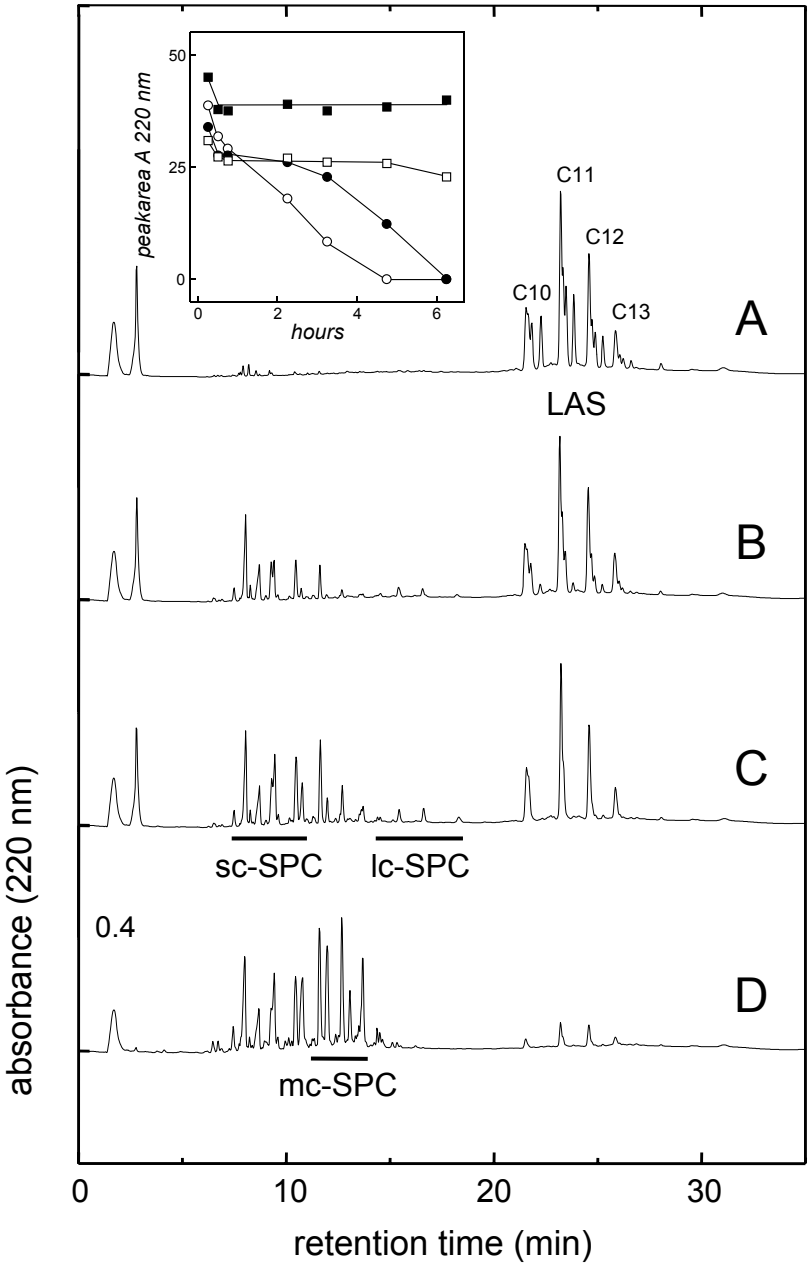

FIG. 4. HPLC chromatograms of samples taken from culture medium in time intervals after addition of LAS to an acetate-growing culture (cf. Fig. 3). Samples were taken after 0.25 h (A), 2.25 h (B), 6.25 h (C) and 68 hours (D). The sets of LAS-peaks of chain-length homologues are stated (C10-C13), in which the respective centrally-substituted congener was the first to elute within each set. The major SPCs (Schleheck *et al.* 2003c) are indicated in groups according to their chain length as short-chain SPC (sc-SPC, C4-C6), medium-chain SPC (mc-SPC, C7-C9), and long-chain SPC (lc-SPC, C10-C13). The peak at retention time 2.5 min represented acetate. The inset shows the detailed analysis of the degradation of e.g. the C10-LAS homologues 2-C10-LAS (❍), 3-C10-LAS (●), 4-C10-LAS (□), and 5-C10-LAS (■), within the first 6 hours after addition of LAS.

We questioned if strain DS-1^T degraded LAS prior to the utilisation of acetate when inoculated to acetate-salts medium with additional LAS present (Schleheck *et al.* 2003e): the organism grew here only in presence of glass particles, and the loss of foaming of the culture medium was observed in the early growth phase two days before the cultures were outgrown. Strain DS-1^T was inoculated from LAS-salts medium into LAS-acetate salts medium, and the disappearance of substrates and the formation of products was followed during growth (Fig. 5). In the first phase of growth, the organism utilised LAS with concomitant formation of SP(d)C, whereas acetate was not utilised; after LAS was almost completely degraded to SP(d)C (no foam), the organism utilised acetate for growth.

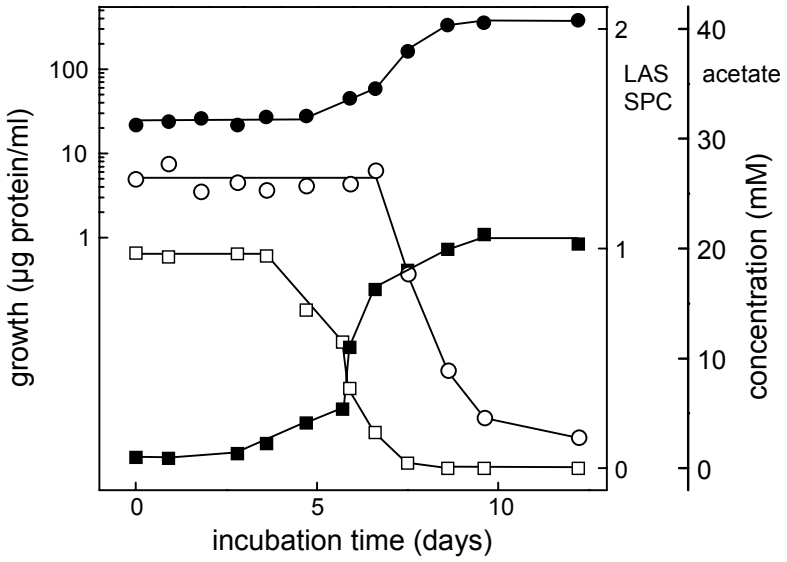

FIG. 5. Growth of strain DS-1 in acetate-salts medium in presence of LAS. The salts medium contained acetate (30 mM), LAS (1 mM), and glass particles (1 mg/ml) to support growth, and was inoculated with LAS-growing cells (1 % v/v). Acetate was in excess presumably due to some limitation (oxygen, trace elements). Symbols: ●, total protein; ❍, acetate; □, total LAS; ■ total SP(d)C.

When strain DS-1^T was inoculated from acetate-salts medium into LAS-acetate salts medium, both compounds were utilised simultaneously (not shown).

The catabolism of LAS is thus apparently dominant, using constitutively expressed enzymes, whereas the acetate catabolism is presumably inducible. Acetate-grown cells, which can be handled easily, are thus a convenient source of degradation enzymes for LAS (see below).

Oxygenase activity detected in whole cells

Whole cells of strain DS-1^T grown with LAS or acetate exhibited substrate-dependent oxygen uptake after addition of LAS when tested, and after addition of linear hexadecane-diphenyletherdisulfonate surfactant (LADPEDS, Schleheck *et al.* 2003d), 4-(1-*n*-octyl)-benzenesulfonate (OBS), dodecane sulfate (SDS), but not after addition of 4-ethylbenzene-sulfonate (Table 1). Octane added in ethanolic solution to LAS-grown cells caused comparatively high oxygen uptake, whereas the direct addition of octane (2 µl) caused only low rates, and the addition of ethanol caused no uptake; ethanol (or acetate) added to acetate-grown cells caused, presumably, respiratory oxygen uptake, but the rate was higher when ethanolic octane was added (Table 1).

The substrate-specific activities determined in acetate-grown cells showed an identical pattern of values of each 50 – 60 % of the respective value determined in LAS-grown cells, and thus confirmed that we were following the same enzyme activity in acetate-grown whole cells. Furthermore, the pattern of substrate-specific activities displayed by LAS- and acetate-grown whole cells reflected the `distance principle´ (see above): the values determined with single LAS congeners with C5-, C8-, or C10-side chain were about 25 %, 85 %, or 90% of the value determined with a congener with C12-side chain (see Table 1).

When LAS was added at concentrations > 100 µM, we observed lysis of the cells, so 80 µM was used routinely. We could not detect LAS under these conditions, and we presumed that the substrates sorbed to the biomass in these dense-cell suspensions (cf. Fig. 3B). Nevertheless,

products were obtained from LAS (see below), LADPEDS, and OBS, and negative controls gave no products. LAS or OBS, added to cell suspensions incubated under anoxic conditions (N_2-gas phase), sorbed readily, but no formation of products was detectable up till air was added to the reactions (data not shown). The initial LAS-degradative reaction was thus dependent on molecular oxygen, which further confirmed that we were following the LAS ω-oxygenase enzyme in whole cells of strain DS-1^T.

Substrate-dependent oxygen uptake was furthermore measurable when other surfactants were tested, which serve as growth substrate for strain DS-1^T (Schleheck *et al.* 2003e) (Table 1), and suggested that the ω-oxygenase converts also these surfactants; we were not able to determine the substrate turnover or product formation for these surfactants. When the obtained substrate-specific activities were compared, the `distance principle´ appeared to be expanded on this wide range of anionic and nonionic surfactants: the values determined for OBS (C8), alkylpolyglucoside (C8), or fatty-acid glucosamide (C10) were comparatively low, and the values were higher when surfactants with long, linear alkyl chain were tested (see Table 1). The apparent exception from this principle was the value determined for LADPEDS (C16) (Table 1): we presume that the ω-oxygenase activity in whole cells is not only limited when a substrate with shorter alkyl chain is converted, but also when a surfactant with a large, bulky, hydrophilic moiety is degraded, and limited transport can be assumed to be responsible for this observation.

TABLE 1. Substrate-specific oxygen uptake rates detected in whole cells of strain DS-1^T.

Substrate added to oxygen electrode and surfactant type (if any)		Growth substrate for strain DS-1^T	Specific oxygen uptake rate of whole cells (mkat/kg protein)	
			Acetate-grown cells	LAS-grown cells
LAS (sidechain $C_{10} - C_{13}$)	A	+	0.48 [a,b]	0.88 [a,b]
2-C10-LAS	A	+	0.42 [a]	0.78 [a]
5-C10-LAS	A	+	0.12 [a]	0.20 [a]
2-C12-LAS	A	+	0.45 [a,b]	0.82 [a]
2-C14-LAS	A	+	0.48 [a]	0.91 [a]
Linear hexadecanediphenyletherdisulfonate	A	+	0.11 [a]	0.32 [a]
4-(1-*n*-Octyl)benzenesulfonate	A	+	0.37 [a,b]	0.62 [a]
4-Ethylbenzenesulfonate	-	-	< 0.01	< 0.01
1-*n*-Dodecanesulfonate	A	+	0.42	*n.d.*
sec-Alkanesulfonate ($C_{14} - C_{17}$)	A	+	0.44	*n.d.*
α-Olefinsulfonate ($C_{14} - C_{16}$)	A	+	0.56	*n.d.*
Methylestersulfonate ($C_{14} + C_{16}$)	A	+	0.48	*n.d.*
Dodecane sulfate	A	+	0.60	1.08
Methyloleoyltaurate	A	+	0.50	*n.d.*
Cholate	A	-	< 0.01	*n.d.*
Alkaneethoxylate	N	+	0.55	*n.d.*
Alkylpolyglucoside (C_8)	N	+	0.11	*n.d.*
Fatty acid glucosamide (C_{10})	N	+	0.25	*n.d.*
Octane	-	+	~ 0.6 [c]	1.6 [c]
Acetate	-	+	0.36 [d]	*n.d.*
Ethanol	-	+	0.19 [d]	< 0.01

[a] Substrate degradation and product formation was confirmed by HPLC.

[b] Whole cells under anoxic conditions (N_2) did not convert the substrate to products, but upon addition of air.

[c] Substrate was added to the oxygen electrode in ethanolic solution.

[d] Respiratory oxygen uptake was detected after addition of ethanol or acetate.

n.d. Not determined.

Oxygenase activity in crude extract

Membrane-free, crude extract (up to 8 mg protein/ml reaction mixture), which was freshly prepared from active acetate-grown cells, showed no substrate-dependent oxygen uptake when e.g. LAS, 2-C12-LAS or OBS was added, and NADH or NADPH was present. The additional presence of FAD, FMN or Fe^{2+} had negligible effect. No substrate disappearance or formation of a product was detectable when e.g. 2-C12-LAS or OBS was added to crude extract with membranes present (up to 13 mg protein/ml reaction mixture). The additional presence of FAD, FMN or Fe^{2+} had negligible effect. We tested a possible `peroxy-shunt´ of the ω-oxygenase (e.g. Wolfe and Lipscomb 2003, see also Matsunaga *et al.* 1996), but neither substrate-conversion nor the formation of a product was measurable in crude extract after addition of H_2O_2 (0.05 and 0.2 mM) to reactions with 2-C12-LAS as substrate. We thus assume that the enzyme is inactive in cell extracts, under the conditions we used.

Molecular screening for an AlkB-type alkane hydroxylase gene in strain DS-1^T

We suspected that a non-heme iron monooxygenase enzyme related to the AlkB-type alkane hydroxylase of *Pseudomonas oleovorans* GPo1 may be present in strain DS-1^T. Genomic DNA from strain DS-1^T was used as template for polymerase-chain reactions (PCR) with highly degenerated oligonucleotides as primers, which were proven to amplify *alkB*-related genes in gram-negative bacteria (see Smits *et al.* 1999). The primers and genomic DNA from *P. oleovorans* GPo1 as a template for positive-control reactions were kindly supplied by J. B. van Beilen, ETH Zürich, Switzerland. The PCR reactions with *P.-oleovorans* DNA as template yielded the specific 558-bp fragment when observed on agarose gels, together with several unspecific fragments (Smits *et al.* 1999); the PCR reactions with strain-DS-1 DNA yielded only

the unspecific fragments, but no 558-bp fragment (not shown). We thus found no evidence to support the presence of a presumed AlkB-type alkane monooxygenase in strain DS-1^T.

Inhibition of the ω-oxygenase in whole cells

$CuSO_4^{2-}$, a specific inhibitor of soluble methane monooxygenase (sMMO) of *Methylococcus capsulatus* (Stirling and Dalton 1980; Green *et al.* 1985), had no effect when tested (2 mM). Methimazole, a competitive inhibitor of flavin-dependent monooxygenases, showed no effect when tested at inhibitory concentration as stated in the literature (0.25 mM, see Horn *et al.* 2003), but some 15 % of reduction was detected when tested at higher concentration (8 mM) (data not shown). When the specific cytochrome P450 inhibitor metyrapone (Testa and Jenner 1981) was tested, the LAS-dependent oxygen uptake of acetate-grown whole cells was reduced in an concentration dependent manner (Fig. 6A): more than 90 % of the activity was permanently lost when 8 mM metyrapone was added to the cells one minute prior to substrate addition (4.4 μmol metyrapone/mg protein). Untreated cells in an scaled-up reaction generated 3-C4-SPC as product after addition of 2-C12-LAS (Fig. 6B); a specific degradation rate of 0.02 mkat/kg could be calculated. No formation of 3-C4-SPC was detectable when 2-C12-LAS was added to metyrapone-treated cells (Fig. 6B). We thus concluded that the ω-oxygenase reaction is inhibited by metyrapone, and that a cytochrome P450-heme is presumably involved in the catalysis.

Cytochrome P450 detected in crude extract

Cytochrome P450-heme containing enzymes can be detected in crude extract due to the different absorption characteristic of oxidised or carbon-monoxide bound P450-heme, and when analysed photometrically as difference spectrum *versus* a reference sample from which molecular oxygen has been removed by addition of dithionite to generate reduced P450-heme

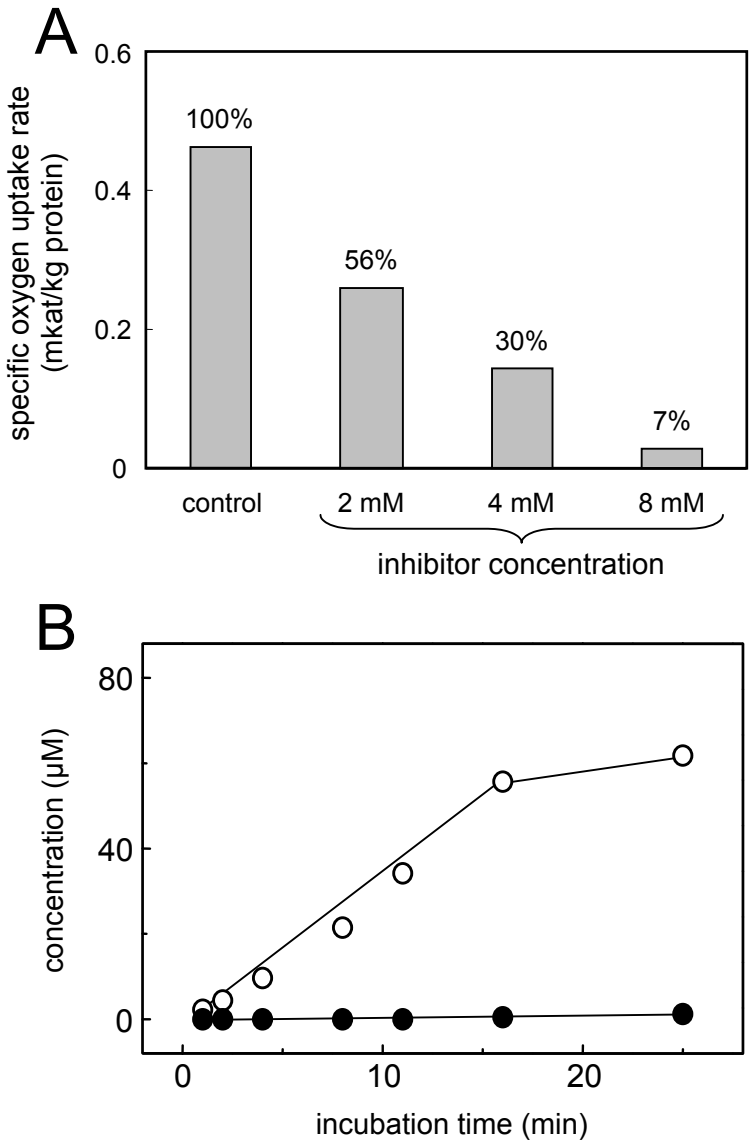

FIG. 6AB. LAS-dependent oxygen uptake rate (A) and the formation of 3-C4-SPC from 2-C12-LAS (B) in dependence on the addition of metyrapone to acetate-grown whole cells. The inhibitor was added one minute before the reactions were started by addition of LAS (A); the value of specific activity relative to the activity of the untreated reaction (control) is stated. During the reactions with 2-C12-LAS (80 µM), the formation of 3-C4-SPC was followed by HPLC in samples taken from the reaction mixture (B); 2-C12-LAS was not detectable during the reactions due to sorption to the biomass; ❍, product formation by untreated cells; ●, product formation by cells treated with metyrapone three minutes prior to substrate addition.

(e.g. Peterson *et al.* 1992). We used crude extract of acetate-grown cells with the membranes present.

An absorption maximum at 406 nm was detected (Fig. 7A) when the spectrum between a sample of oxic extract *versus* a sample of reduced extract was compared; this absorption maximum was absent in the spectrum taken between a sample of reduced and carbon-monoxide saturated extract *versus* a reference sample with reduced extract: a new maximum appeared at 428 nm (Fig. 7A). After the membranes were removed (50,000 g, 30 min), most of the absorption at 406 nm (oxic-*versus*-reduced) was detected in the soluble sub-cellular fraction (Fig. 7B), and not when the particulate fraction was solublised with SDS (0.5 %), and analysed.

The observed absorption maxima differed from that of other P450s only in the exact location: the absorption maximum of e.g. the carbon-monoxide bound P450-heme is usually observed at around 450 nm wavelength (`P450´), and we thus have detected a cytochrome `P428´-containing, soluble enzyme in strain DS-1^T.

The observed absorbance of the carbon-monoxide bound heme (Fig. 7A) allowed the amount of P450-heme present in the crude extract to be estimated at about 0.15 nmol/mg of protein, when calculated with an assumed ε of the carbon-monoxide bound P450-heme of 91 1/(mM*cm) (Peterson *et al.* 1992), and the protein concentration we measured (7 mg protein/ml). This is a relatively high value (cf. Asperger *et al.* 1986; Peterson *et al.* 1992).

We consider it as very likely that this enzyme is involved in the catalyses of the ω-oxygenation of LAS in strain DS-1^T, due to the observed inhibition of ω-oxygenase activity in whole cells (Fig. 6) by the P450-specific ligand metyrapone (Testa and Jenner 1981). The observation of the P450-like protein in extract from (non-induced) acetate-grown cells, which displayed ω-oxygenase activity, in combination with the fact that bacterial P450-heme oxygenases are comparatively rare in bacteria when compared to the vast array of known (multible) P450s from eukaryotic sources, supports this consideration, as did the absence of evidence for an *alk*B-type non-heme iron hydroxylase gene in strain DS-1^T (PCR-experiments).

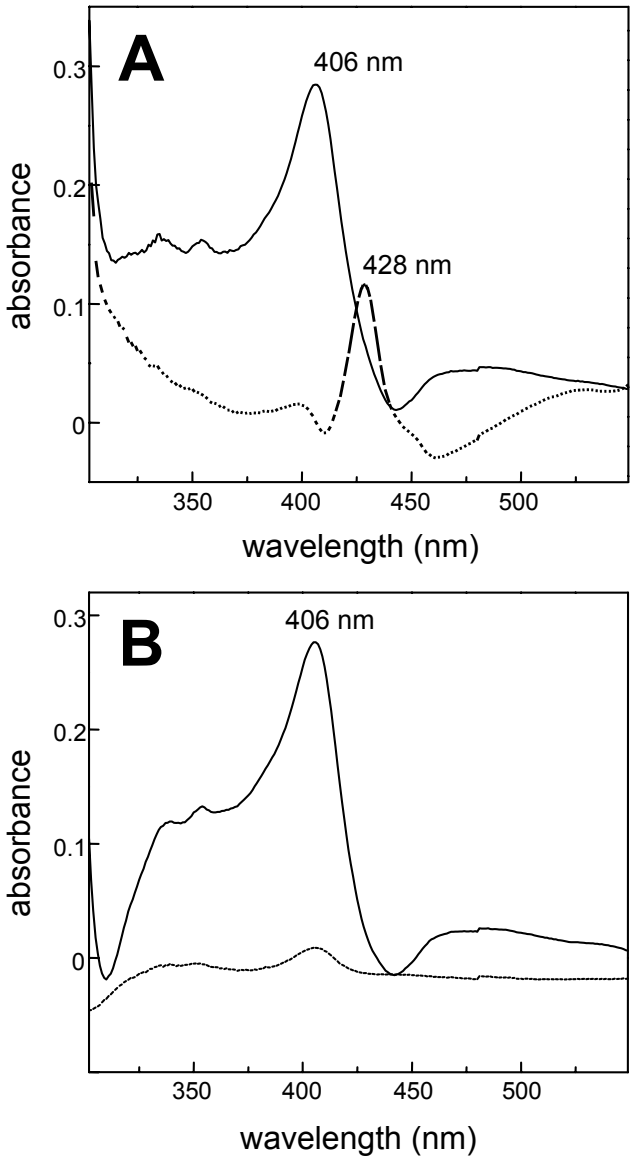

FIG. 7. Difference spectra of cell extract from acetate-grown strain DS-1^T cells. The spectrum of oxic extract (solid line in A) and of CO-saturated dithionite-reduced extract (dashed line in A) was recorded against a reference sample of dithionite-reduced extract, each with the membranes present. The membranes were removed by ultracentrifugation, and the spectrum of the oxic soluble fraction (solid line in B) and of the oxic solublised membrane fraction (dotted line in B) was recorded against respective dithionite-reduced reference samples.

The precedent for presumed P450 ω-oxygenase in strain DS-1^T is the characterised NonM- (CYP153-) oxygenase, which catalyses the ω-oxygenation of *n*-alkanes in *Acinetobacter* sp. (Maier *et al.* 2001), and is constituted in an multi-component oxygenase system with electron transfer components to deliver electrons from NADPH/NADH to the P450-heme. The behaviour of measurable specific activity of LAS-oxygenation during growth (Fig. 2) suggested that the ω-oxygenase enzyme in strain DS-1^T is contributed by several components. Nonetheless, NonM is distributed in cell-free extracts among particulate and soluble sub-cellular fractions (Müller *et al.* 1989), whereas the presumed P450-oxygenase in strain DS-1^T was apparent almost exclusively in the soluble fraction.

We thus postulate a soluble, multi-component, P450-dependent oxygenase system in strain DS-1^T, which converts LAS and other surfactants, as well as alkanes, into hydroxylated derivatives, which are then oxidised and undergo chain-shortening through β-oxidation (cf. Fig. 1). The enzyme activity was apparently lost when strain DS-1 cells were disrupted to prepare crude extract, but we hope to be able to further characterise the enzyme when we follow the absorption characteristic of this presumed oxygenase (component) during protein-purification steps.

ACKNOWLEDGEMENTS

J. B. van Beilen kindly supplied the *alkB* PCR-primers and *Pseudomonas oleovorans* DNA, and C. Steber and C. Groß helped on growth experiments during students research courses. Funds were made available by the University of Konstanz, the Stiftung Umwelt und Wohnen, and ECOSOL.

CHAPTER 4

Desulfonation and Degradation of the Disulfodiphenylethercarboxylates from Linear Alkyldiphenyletherdisulfonate Surfactants

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ABSTRACT

Earlier work showed that the biodegradation of a commercial linear monoalkyldiphenyletherdisulfonate (LADPEDS) surfactant as a carbon source for microbial growth leads to the quantitative formation of corresponding disulfodiphenylether carboxylates (DSDPECs), which were not degraded. *Parvibaculum lavamentivorans*^{T} DS-1 catalyzes these reactions. These DSDPECs have now been characterized by high pressure liquid chromatography coupled *via* an electrospray interface to a mass spectrometer. DSDPECs were a complex mixture of compounds which indicated catabolism *via* ω-oxygenation and β-oxidation. DSDPECs were subject to quantitative desulfonation in bacterial cultures in which they served as sole sulfur sources for bacterial growth. On average, one sulfonate group per DSDPEC species was removed, and the organism responsible for this desulfonation was isolated and identified as *Rhodococcus opacus* ISO-5. The products were largely monosulfodiphenylethercarboxylate-phenols (MSDPEC-phenols). MSDPEC-phenols were subject to extensive dissimilation by bacteria from activated sludge.

INTRODUCTION

The linear monoalkyldiphenyletherdisulfonate surfactants (LADPEDS; Fig. 1) have been in use for some 40 years in industrial processes (Quencer and Loughner 2001), which include the production of synthetic latex, and the uses of the latter in carpet production, paints and paper coatings (Klein 1983), as well as in subsurface remediation (Rouse *et al.* 1993; Sabatini *et al.* 1997; see also Quencer and Loughner 2001). Despite this widespread usage of LADPEDS, and their classification in the USA as biodegradable (Rouse *et al.* 1993), little has been published on their metabolism. It was shown recently, however, that their initial metabolism in pure culture is analogous to that of the linear alkylbenzenesulfonate (LAS) surfactants, namely ω-oxygenation

and oxidation of the side chain followed by ß-oxidation, which results in the release of the correspondingly smaller disulfodiphenylether carboxylate (DSDPEC; Fig. 1), which was not further degraded as a source of carbon for growth (Schleheck *et al.* 2000).

The formation of DSDPECs in pure culture corresponds to a company report on work with a typical [*ring*-^{14}C]-labeled congener of LADPEDS in activated sludge (Quencer and Loughner 2001). Whereas no further metabolism of DSDPECs was detected in the laboratory (Schleheck *et al.* 2000), the company report showed slow release of $^{14}CO_2$ from the [*ring*-^{14}C]-labeled DSDPECs incubated in soil, which indicates ring cleavage reactions in soil (Quencer and Loughner 2001).

It is easy to rationalize the slow dissimilation of the DSDPECs. LADPEDS represents some 210 racemic compounds (legend to Fig. 1). The largest LAS homologue, C13, represents six compounds, five of which are racemic; though they are fully degradable (e.g. Swisher 1987), we suspect the microbiology involved to be fairly complex and to involve enzymes of relatively high specificity (Schleheck *et al.* 2000; Schulz *et al.* 2000). Correspondingly, degradation of the numerous DSDPECs would require orders of magnitude more organisms.

An alternative to large numbers of specific enzymes is a non-specific attack. This is especially useful with organosulfonates, because the general desulfonation of aromatic compounds yields the corresponding phenol (Kertesz 2000), and the hydroxy-analogue of a benzenesulfonate (i.e. the phenol) is generally degradable (Wellens 1990). Recent work indicates that a desulfonation of 'recalcitrant' xenobiotic compounds could lead to their further degradation or binding to soil components (Laue *et al.* 1996; Mampel *et al.* 1998; Rein and Cook 1999; Ruff *et al.* 1999; see also Kudlich *et al.* 1999).

The present work shows that DSDPECs can indeed be desulfonated, and that the products are available for the further biological attack already indicated in soil systems (Quencer and Loughner 2001).

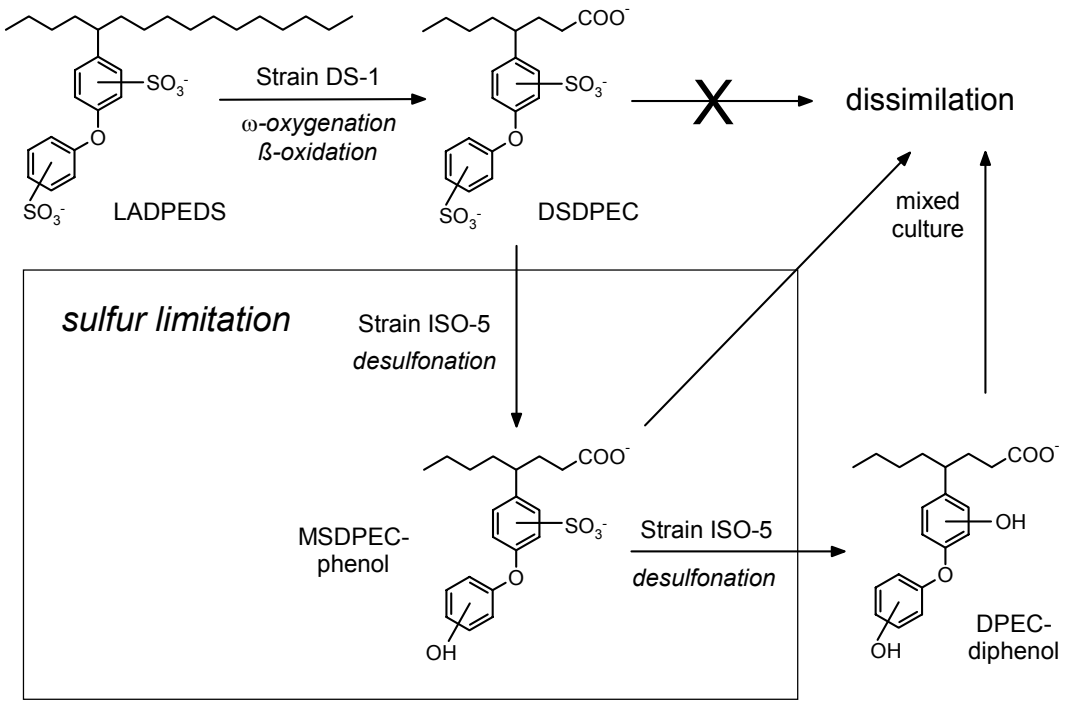

FIG. 1. LADPEDS, its dissimilation to a corresponding DSDPEC by *P. lavamentivorans*^{T} DS-1, the product of its subsequent desulfonation by *R. opacus* ISO-5, the product of a second desulfonation, and putative fate of the residual carbon skeleton. Commercial LADPEDS is nominally a set of compounds with a C16 alkane subterminally substituted with a disulfodiphenylether: there are thus seven positional isomers of the substituent on the chain, each with a chiral center, ten possible combinations of alkyl, sulfono and ether substituent on the first ring, and three positional isomers of sulfonation on the second ring. LADPEDS thus represents up to 210 racemic compounds; *meta* substitution of the sulfono substituent relative to the alkyl chain is unlikely. Dialkylated species and monosulfonated ethers also occur (Quencer and Loughner 2001), and several homologues were found in the preparation (see text). The commercial product is thus extremely complex. Strain DS-1^T produces several intermediates per substrate with LAS (Dong *et al.* 2003; Schleheck *et al.* 2003b), and we have now confirmed that this occurs also with LADPEDS. Strain ISO-5 has limited capacity to carry out a second desulfonation, and a mixed culture catalyses extensive dissimilation of MSDPEC-phenols and DPEC-diphenols (see text). The first horizontal line in the figure represents published data (Schleheck *et al.* 2000); the other reactions were observed in this work (see also Quencer and Loughner 2001).

MATERIAL AND METHODS

Materials

LADPEDS, as DOWFAX 8390, was supplied by Dow Chemical Company, Midland, MI, USA (Schleheck *et al.* 2000). DSDPECs were generated as catabolic products from LADPEDS in liquid cultures (1 mM LADPEDS; the molecular weight of the disodium salt is 598, see Schleheck *et al.* 2000) and separated from the growth medium by solid phase extraction (see below). DSDPECs have the same UV-spectrum as the parent LADPEDS (Schleheck *et al.* 2000) and DSDPECs were quantified as having the same molar absorption coefficient as LADPEDS (Schleheck *et al.* 2000). MSDPEC-phenols were generated as desulfonation products from DSDPEC in liquid cultures and separated from the growth medium by semi-preparative HPLC (see below). Commercial linear alkylbenzenesulfonate (LAS) surfactant (Marlon A 350, Hüls, Marl, Germany) was converted to the corresponding sulfophenylcarboxylates (SPC) as described for the conversion of LADPEDS to DSDPEC.

Activated sludge was obtained from the urban sewage treatment plants in Konstanz and Radolfzell, Germany, and from the BASF industrial plant in Ludwigshafen, Germany. The sources of routine chemicals were given elsewhere (Kölbener *et al.* 1994; Laue *et al.* 1996).

Growth media

Two different growth media were used. Carbon-limited, phosphate-buffered, minimal-salts medium was used in the generation of DSDPECs from LADPEDS (Thurnheer *et al.* 1986; Schleheck *et al.* 2000). Sulfur-limited, Tris-buffered salts solution with three sources of carbon in excess (Laue *et al.* 1996) was used to enrich for organisms able to desulfonate DSDPECs. This medium was optimized for work with strain ISO-5: the phosphate concentration was raised to 4 mM and only one carbon source was present, 25 mM glucose.

Enrichment cultures, growth under sulfur-limited conditions and bacteria used

Enrichment cultures to utilize commercial DSDPECs (or LADPEDS) as the sole source of sulfur for growth for microorganisms were done with a 1 % inoculum in 3-ml cultures in 30-ml screw-cap tubes which were aerated at 30 °C in a roller (Zürrer *et al.* 1987; Laue *et al.* 1996). Cultures were considered positive when both growth and substrate disappearance (HPLC) occurred.

Molar growth yields, on the 3-ml scale (to economize on the amount of DSDPECs to be purified), were obtained by inoculating a set of tubes with different concentrations (0 to 50 µM sulfur) of sulfate or DSDPECs and quantifying the protein synthesized. The molar growth yield was the slope of the line obtained by plotting the protein formed against the initial substrate concentration (e.g. Cook 1987).

For experiments to identify products, cells were removed from 100-ml cultures by centrifugation (10,000 g, 30 min, 4 °C). The supernatant fluid was brought to pH 2 with 1 M HCl and passed through a membrane filter of 0.2-µm pore diameter. The acidified medium involved in the generation of DSDPEC was diluted to about 0.1 mM DSDPEC for analyses by LC-MS; that for the generation of MSDPEC-phenols concentrated by evaporation to about 60 µM MSDPEC-phenol.

Strain ISO-5 was grown in 300 ml of medium with 50 µM DSDPEC as sulfur source to generate MSDPEC-phenols for the degradation test, and cells were removed by centrifugation (10,000 g, 30 min, 4 °C). The supernatant fluid was acidified to pH 2 with 1 M HCl and a 50-ml portion loaded on to a semi-preparative, reversed phase column (Beckman ULTRASPHERE ODS, 10 x 250 mm, 5-µm particle size) that was equilibrated with 4 mM phosphate buffer, pH 2 (buffer A) at 5 ml/min. The column was then washed for 10 min with buffer A. Bound material eluted in one broad peak after the methanol concentration was ramped to 100 % in 2 min; this material was collected in a 15-ml fraction. Two collected fractions were pooled and evaporated to dryness at 40 °C in a rotary evaporator, and dry material was dissolved in 10 ml of carbon-limited salts medium. This MSDPEC-phenol salts medium was inoculated with 0.2 ml sludge derived from a communal sewage treatment plant (Konstanz, Germany); prior to inoculation, the activated sludge was repeatedly washed and resuspended in carbon-limited salts medium. The culture was incubated at 30 °C on an orbital shaker, and samples for analyses by HPLC and for the quantification of protein were taken at intervals.

Parvibaculum lavamentivorans^{T} DS-1 (DSM 13023^{T}, NCIMB 13966^{T}) (Schleheck *et al.* 2000; Schleheck *et al.* 2003e) was grown under carbon-limiting conditions throughout the work. *Pseudomonas putida* S-313 (DSM 6884) was grown under sulfur-limited conditions with Orange II as the sole added source of sulfur (Kertesz *et al.* 1994).

Analytical methods

Commercial LADPEDS, DSDPECs, MSDPEC-phenols, and the compounds tested as sulfur sources for strain ISO-5 were routinely determined by gradient elution with a perchlorate-containing eluent in reversed phase HPLC with 125 x 3 mm columns of Nucleosil-5-C18 (Schleheck *et al.* 2000) (gradient system I). Ion chromatography with suppression was done as described elsewhere (Laue *et al.* 1996).

The HPLC used for LC-MS was a Hewlett Packard Series 1100 (Hewlett Packard Schweiz AG, Urdorf, Switzerland) with a variable wavelength UV detector, set to 220 nm. The HPLC column (150 x 1 mm) used was a Phenomenex, Ultracarb 5 ODS 30 (Brechbuehler AG, Schlieren, Switzerland). Eluent A was aqueous 5 mM acetic acid and 5 mM triethylamine, and eluent B was acetonitrile/water (80:20) containing 5 mM acetic acid and 5 mM triethylamine (Eichhorn and Knepper 2002) (gradient system II). The flow rate was 100 µl/min, and the column was maintained at 30 °C. The column was equilibrated with eluent containing 95 % A and 5 % B. Samples (100 µl) were injected and a linear gradient to 100 % B in 35 min was applied: after another 5 min, the initial conditions were regenerated in 5 min, with an

equilibration phase of 5 min, giving a total run time of 50 min. All mass spectra were acquired on a Platform LC single quadrupole mass spectrometer, using electrospray ionization (Micromass UK Ltd., Manchester, UK). Full scan spectra were acquired in negative ion mode, scanning from *m/z* 50 - 800 at 1 s/scan. The mass range was calibrated and the sensitivity of the instrument tested using 2 mM $NaNO_3$ infused at a flow rate of 70 µl/min. The electrospray interface temperature was set to 150 °C, and the flow of nitrogen gas to 500 l/h. The needle- and cone-voltages were set to 4 kV and 60 V, respectively.

Solid phase extraction was done on a column of Chromabond HR-P (2 g; Macherey & Nagel, Düren, Germany) (Mampel *et al.* 1998); extraction and recovery were quantitative, and the contamination by sulfate was undetectable, as observed previously (Mampel *et al.* 1998). Protein in bacterial cells was assayed by a Lowry-type method (Cook and Hütter 1981). A partial 16S-rRNA gene sequence (450 bp) of strain ISO-5 was determined by the German Culture Collection (DSMZ, Braunschweig, Germany), where the sequence data were aligned and compared as described elsewhere (Rainey *et al.* 1996; Maidak *et al.* 2001).

RESULTS

Generation of DSDPECs from commercial LADPEDS

LADPEDS was previously detected in dried solutions by matrix-assisted laser-desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) (Schleheck *et al.* 2000), but new separative techniques (Eichhorn and Knepper 2002) allowed LC-MS to be used to examine the starting material for these experiments. LADPEDS was separated in gradient system II, and the major signals obtained, from 25-35 min, were C16-LADPEDS. They were recognized as *m/z* = 553 $(M-H)^-$, *m/z* = 276 $(M-2H)^{2-}$, *m/z* = 575 $(M+Na-2H)^-$, and *m/z* = 654 $(M+(H_5C_2)_3N-H)^-$, together with the desulfonated fragment *m/z* = 473 $(M-H-SO_3)^-$. There were significant amounts of LADPEDS with chain lengths C10, C11 and C12, and traces with chain lengths C13, C14, C18 and C20. The second major signal was near 34 min, where *m/z* = 473 $(M-H)^-$, and this was presumed to be the monosulfonated diphenylether referred to in the manufacturer's description (legend to Fig. 1). The dialkyl compounds (legend to Fig. 1) would have much longer retention times and were not observed under these conditions.

LADPEDS was subject to partial dissimilation of the alkyl chain by *P. lavamentivorans*^{T} DS-1 (Schleheck *et al.* 2003e). When this phenomenon was first described, only partial IR spectra were available with which to deduce the identity of DSDPEC (Fig. 1), together with MALDI-TOF-MS identifications of the analogous product(s) from LAS (Schleheck *et al.* 2000). We then realized that we had underestimated the number of products from each LAS congener (Schleheck *et al.* 2003b; Schleheck *et al.* 2003c; see also Eichhorn and Knepper 2002). Correspondingly, the LC-MS analyses of putative DSDPEC showed a wide range of compounds (Table 1).

The major compound formed from LADPEDS was the C8-DSDPEC, the chain length anticipated from physiological data (Schleheck *et al.* 2000); the second major compound was

C6-DSDPEC. These 'compounds', however, were really large groups of compounds which eluted over some fifteen minutes, analogous to the 'educt' being nominally 210 racemic compounds (Fig. 1, legend). Lower amounts of C10-DSDPEC were also detected, as well as readily measurable amounts of C7-DSDPEC, and traces of C5-, C9- and C11-DSDPEC.

TABLE 1. Products from the dissimilation of commercial LADPEDS by *P. lavamentivorans*^{T} DS-1 detected by LC-MS [a].

Length of side chain [b]	Monocarboxylated disulfonate		Monocarboxylated monosulfonate	
	$(M-H)^{-}$ [c]	Intensity	$(M-H)^{-}$ [c]	Intensity
4	415	ND	335	+
5	429	tr	349	tr
6	443	+ + +	363	+ +
7	457	+	377	+
8	471	+ + +	391	+ +
9	485	tr	405	tr
10	499	+	419	tr
11	513	tr	433	ND
12	527	ND	447	ND
13	541	ND	461	ND

[a] The abundant compounds were recognized from the pattern of peaks for $(M-H)^{-}$, $(M-2H)^{2-}$, $(M+Na-2H)^{-}$, and $(M+(H_5C_2)_3N-H)^{-}$, together with the desulfonated fragment $(M-H-SO_3)^{-}$; the regularity in the pattern of elution of homologues allowed traces to be identified. Gradient system II was used for the separation.

[b] Number of C atoms.

[c] Data (*m*/*z*) from the peaks for $(M-H)^{-}$ are given: the signals were spread over several minutes, presumably reflecting the complexity of the parent surfactant (see legend to Fig. 1).

ND, not detected.

tr, trace detected.

+, + + and + + +, signal intensities.

The mean chain length (about C8) thus corresponds to that predicted earlier (Schleheck *et al.* 2000). The mass spectral identifications also confirm the prediction of the identity of the family of products, i.e. DSDPECs. The monosulfonated components of commercial LADPEDS were also subject to dissimilation, to products with a similar range of chain lengths (Table 1). No unsaturated species (cf. Eichhorn and Knepper 2002; Dong, Knepper and Cook, unpublished), and no significant amounts of dicarboxylated LADPEDS (cf. Dong *et al.* 2002; Schleheck *et al.* 2003b) were detected. For convenience, this whole group of products is termed DSDPEC.

Enrichment cultures

Enrichment cultures to degrade 100 μM commercial LADPEDS as the sole added source of sulfur with inocula from sewage works gave slight growth. Commercial LADPEDS was found by ion chromatography to contain inorganic sulfate, giving 10 μM sulfate in the growth medium. LADPEDS, which had been purified of this contamination by solid phase extraction, supported no growth.

Enrichment cultures to degrade 60 μM DSDPECs as the sole added source of sulfur for growth were prepared. Of the three inocula tested, only that from the industrial sewage treatment plant was positive. This enrichment culture was largely homogeneous when examined microscopically after three subcultures in fresh medium, and the major morphotype was coryneform. Coryneform strain ISO-5 was isolated from the culture, and it catalyzed the same reactions(s) (see below) as the enrichment culture. The 16S-rRNA gene of strain ISO-5 was subject to partial sequencing, and the sequence was identical with that of *Rhodococcus opacus*^{T} (DSM 43205), a typical coryneform organism (Stackebrandt *et al.* 1997). Our isolate was thus *Rhodococcus opacus* ISO-5, which was deposited with the German Culture Collection (DSMZ, Braunschweig, Germany) as DSM 44600.

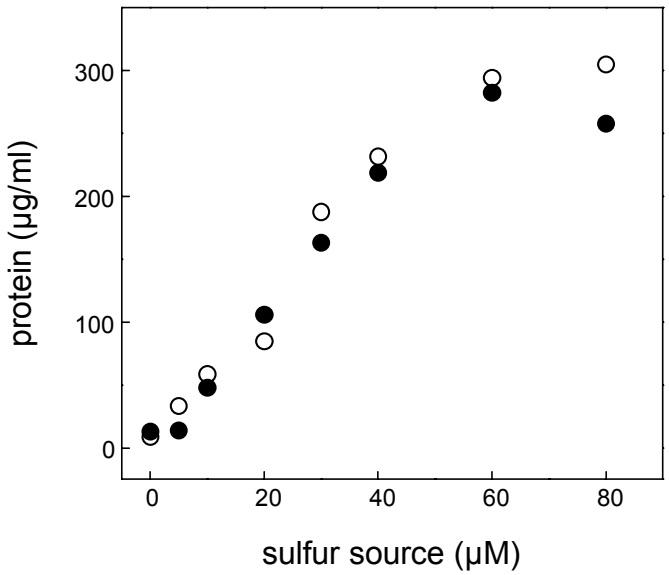

FIG. 2. Growth yield of *R. opacus* ISO-5 in salts medium with different limiting concentrations of sulfate (○) and of DSDPEC (●).

DSDPEC and other compounds as sulfur sources by *Rhodococcus opacus* ISO-5

Strain ISO-5 utilised sulfate with a molar growth yield of 5 kg protein/mol S up to about 40 µM sulfate (Fig. 2), a value which corresponds to complete incorporation into cell material (Kertesz 2000). The growth yield with DSDPEC was also 5 kg protein/mol (Fig. 2), so strain ISO-5 removed an average of one sulfonate group per DSDPEC species. Growth with DSDPEC was concomitant with disappearance of individual compounds during growth (Fig. 3, inset), with a steady shift of peaks in HPLC determinations to longer retention times (Fig. 3) and the formation of individual products was concomitant with growth (Fig. 3, inset). Each product peak had a UV spectrum different from that of the substrate (not shown), which was a first indication that the aromatic ring was subject to microbial attack. The products were examined by LC-MS (Table 2). MSDPEC-phenols (Fig. 1) were detected. The major set represented the chain length C8 (Table 2) and it was spread over several minutes, as were the precursors (cf. Table 1); further, individual peaks with other chain lengths were observed (Table 2). DPEC-diphenols were also

detected (Fig. 1, Table 2), which indicates that some species were subject to a second desulfonation: these products were individual sharp peaks, rather than the broad sets of compounds in the precursors (Table 1).

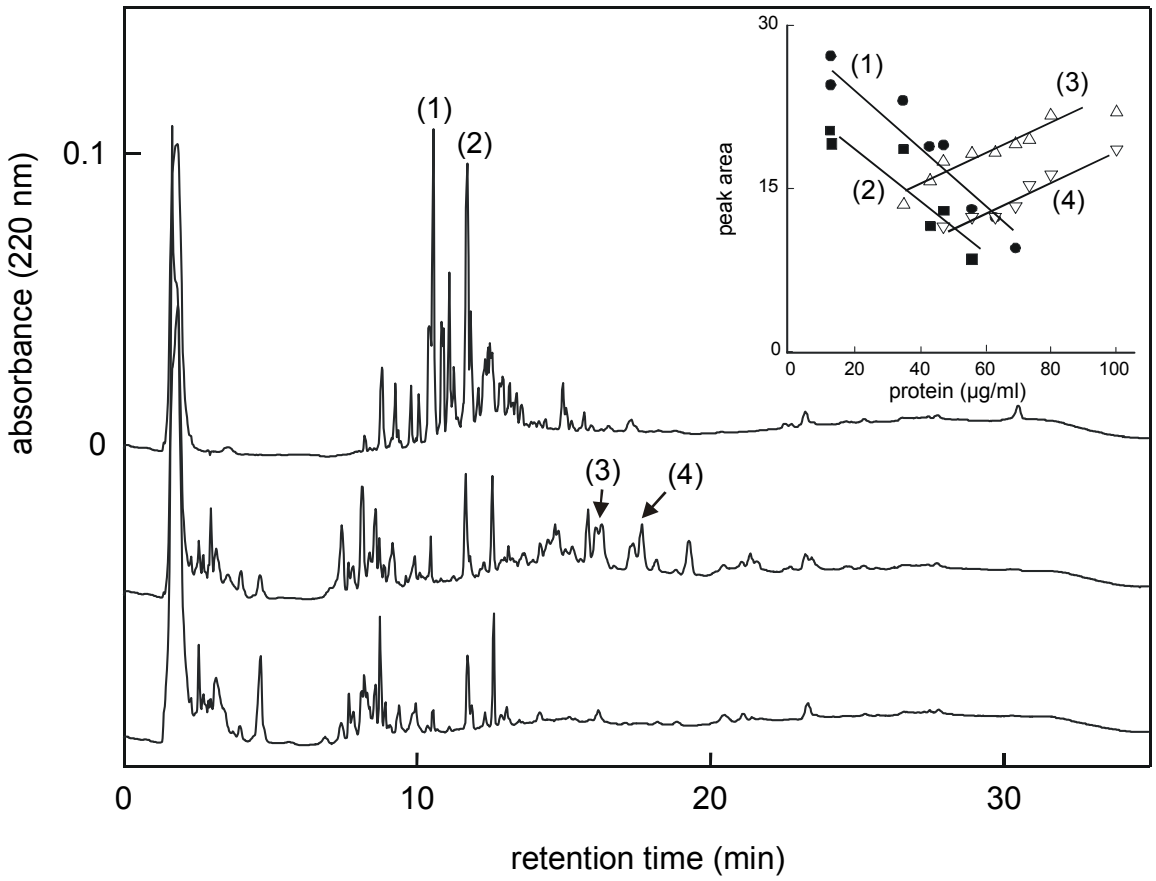

FIG. 3. HPLC chromatograms of DSDPEC in sulfur-limited salts medium (upper chromatogram), of the same medium after growth of *R. opacus* ISO-5 (central chromatogram) and of outgrown sulfate-salts medium (lower chromatogram). Gradient system I was used. The peaks visible in the negative control (lower chromatogram) are natural excretion products from strain ISO-5, so the peaks with retention time < 13 min in the central chromatogram are the same excretion products from strain ISO-5. Inset is a plot of disappearance of individual peaks as a function of growth and appearance of products as a function of growth.

TABLE 2. Products from the desulfonation of DSDPEC by *R. opacus* ISO-5 detected by LC-MS [a].

Length of side chain [b]	MSDPEC-phenol [c]		DPEC-diphenol	
	$(M-H)^-$	intensity	$(M-H)^-$	intensity
4	351	+	287	ND
5	365	tr [d]	301	tr [d]
6	379	tr [d]	315	+ + [d]
7	393	ND	329	+ [d]
8	407	+ +	343	+ + [d]
9	421	tr	357	ND
10	435	tr	371	ND

[a] Gradient system II was used for the separation.

[b] Number of C atoms.

[c] Data (*m*/*z*) from the peaks for $(M-H)^-$ are given: unless otherwise indicated, the signal was spread over several minutes, presumably reflecting the complexity of the parent surfactant (see legend to Fig. 1).

[d] In contrast to the many broad peaks observed, especially in Table 1, this peak is sharp and seemingly represents only one or a few chemical species.

ND, not detected.

tr, trace detected.

+ and + +, signal intensities.

The complexity of the range of DSDPECs desulfonated led us to explore the substrate range of the organism. Commercial LAS was converted by strain DS-1^T into sulfophenylcarboxylates (SPC) (Schleheck *et al.* 2000), and strain ISO-5 grew with SPCs as longer retention times after growth, different UV spectra from the educts, and mass spectra which sole source for sulfur: as observed with LADPEDS, HPLC-peaks of desulfonated SPCs shifted to showed these compounds to be desulfonated to the corresponding phenols. The chain length was not significantly altered during the desulfonation (data not shown). Strain ISO-5 utilised benzenesulfonate, 4-toluene-, 4-ethylbenzene-, 4-octylbenzene-, 4-phenol-, 4-chlorobenzene-, 2-, 3- and 4-aminobenzene- and 2-, 3- and 4-nitrobenzenesulfonate, 4-, and 3-

(but not 2-) sulfobenzoate, 5-sulfosalicylate, 5-amino-2-chlorotoluene-4-sulfonate, naphthalene-2-sulfonate, anthraquinone-2-sulfonate, 8-amino-1,5-naphthalenedisulfonate, and the dyestuffs orange II, roccelline, Ponceau R, Erichrome red B, azophloxine and tartrazine, but not amaranth, Ponceau S or Congo red as sole sulfur sources for growth.

The product tended to bind to the organism, which was especially visible with the dyestuffs when outgrown cultures were centrifuged. In the case of orange II (and tartrazine), the desulfonation product from the cell pellets was extracted into alcohol and examined by HPLC. The product was indistinguishable from the product from orange II (and tartrazine) generated by *P. putida* S-313 and identified previously (Kertesz *et al.* 1994).

Dissimilation of MSDPEC-phenol

The products of desulfonation of DSDPEC (MSDPEC-phenol; Fig. 3) were separated from growth medium by semi-preparative HPLC and supplied as sources of carbon in what we regarded as a miniaturised OECD 301 biodegradation test; the natural excretion products from strain ISO-5 were also present in this preparation (cf. Fig. 3).

We observed growth as turbidity, largely due to rod-shaped bacteria, and substrate disappearance (Fig. 4), whereas the negative control, i.e. without MSDPEC-phenol, showed no growth. Within the first five days of incubation, the natural excretion products from strain ISO-5 were mostly degraded (retention time < 13 min), but only little MSDPEC-phenol (retention time > 13 min). MSDPEC-phenol disappeared in the following fifteen days to about 32 % of the initial, when quantified as total peak area of UV-absorptive material (A220, retention time from 13 to 25 min; Fig. 4); the biomass production over this period was 30 µg protein/ml. We estimated an initial concentration of about 0.5 mM MSDPEC-phenol in the medium, and we assumed a mean sidechain of C8 (Table 2). So the initial concentration of carbon was 10 mM, of which 7 mM disappeared. The molar growth yield was thus about 4 g protein/mol C, a normal value (e.g. Cook 1987).

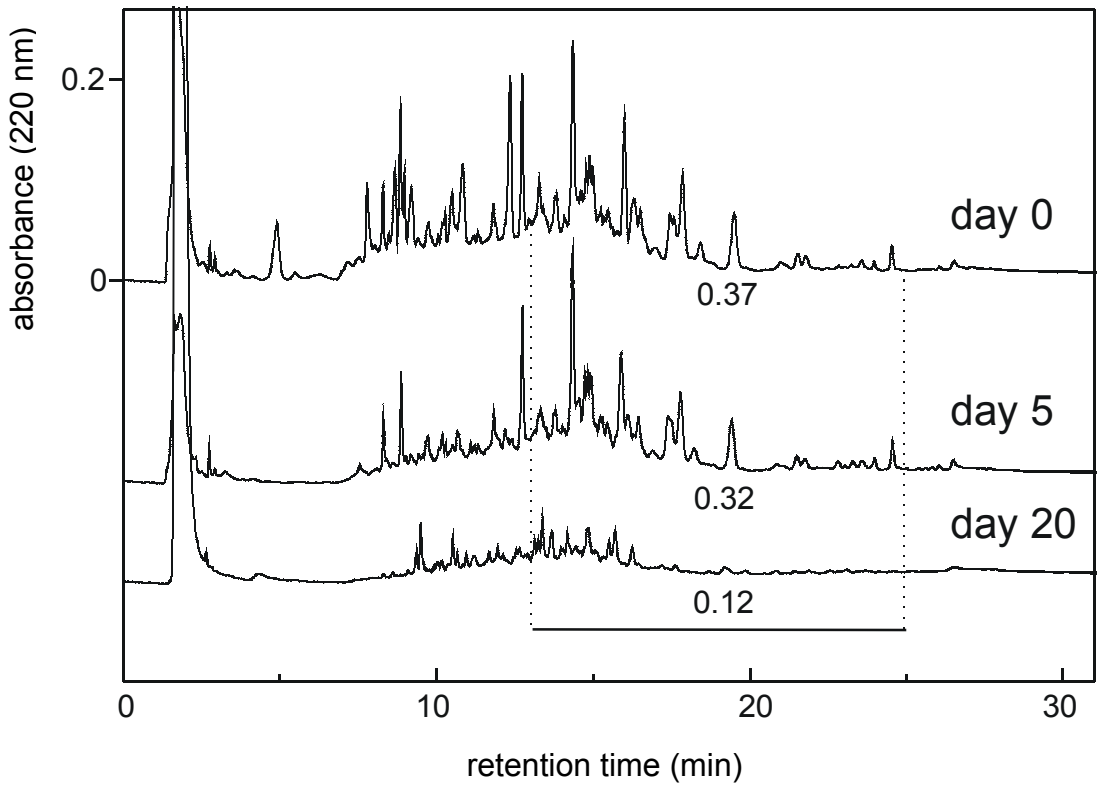

FIG. 4. HPLC chromatograms of the levels of MSDPEC-phenols and DPEC-diphenols during incubation with an inoculum derived from sewage sludge. Gradient system I was used. Total peak area of UV-absorptive material in the interval from 13 to 25 min (bar) is indicated.

DISCUSSION

Basic research with commercial surfactants is often made more complex by nature of the commercial product. This is certainly the case with LADPEDS, where the one structure in Fig. 1 represents some 210 optically active compounds, to say nothing of the homologues and the monosulfonated species that were also detected. There are thus many HPLC peaks for any relevant value of *m/z*. There was good indirect evidence to predict the generation of DSDPEC from LADPEDS (Schleheck *et al.* 2000); these preliminary data have now been confirmed by LC-MS. We initially considered C8-DSDPEC to be the product (Schleheck *et al.* 2000). This predictions was accurate, in that the mean chain length represented about C8-DSDPEC. However, a wide range of chain lengths was found as opposed to a single chain length (Table 1). A further prediction (Dong *et al.* 2002) foresaw that metabolism is due solely to ω-oxygenation and ß-oxidation. Detectable amounts of DSDPEC with sidechains of non-even chain length (Table 1) were observed, but we assume that they derive solely from the C11- and C13-LADPEDS, and not that we have detected the α-oxidation (Finnerty 1988) reported for LAS in major reviews (Swisher 1987; Schöberl 1989).

There was negligible ω-oxygenation of the second methyl group of LADPEDS: in contrast, dicarboxylates were abundant with LAS as the substrate (Schleheck *et al.* 2003b; see also Eichhorn and Knepper 2002). No α,ß-unsaturated derivatives of DSDPEC were detected: low levels of such derivatives were found with LAS as the substrate (Eichhorn and Knepper 2002; Schleheck *et al.* 2003c). Perhaps they were lost in the multiplicity of compounds present.

DSDPECs, incubated under conditions of carbon limitation and excess sulfate, were not subject to microbial attack (Fig. 1); they were also observed to be stable compounds (Schleheck *et al.* 2000). In contrast, when incubated with *R. opacus* ISO-5 in the absence of sulfate and in the presence of an alternate source of carbon, DSDPECs were subject to quantitative

biotransformation (Fig. 3). This biotransformation could be deduced to represent monodesulfonation (Fig. 2), which was largely confirmed by LC-MS (Table 2). Many single desulfonations were detected, giving rise to the corresponding MSDPEC-phenol (Fig. 1, Table 2), as predicted from earlier work (Zürrer *et al.* 1987; Kertesz 2000). Some species of DSDPEC were subject to two desulfonation reactions to give the DPEC-diphenol (Fig. 1, Table 2). We presume that the initially monosulfonated species (legend to Fig. 1) were also desulfonated, but we suspect that the product was sufficiently non-polar to adsorb to the cell (see below) and was thus not detected in the culture fluid.

Strain ISO-5 demonstrated a broad substrate range for sulfonated compounds (Fig. 3, Table 2) so we explored some of its limits. Almost all small compounds tested (benzenesulfonate to 4-(1-octyl)benzenesulfonate), and a range of dyestuffs up to molecular weight 468 (tartrazine) were desulfonated; the larger dyestuffs (above molecular weight 538) were not. Very few of the products were found in solution, and work with the dyestuffs indicated that the apolar products bound to the cell, which presumably reflects the presence of apolar mycolic acids in large amounts at the cell surface, typical of *Rhodococcus* (Prescott *et al.* 1996).

The phenomenon of desulfonation under sulfate-limiting conditions is controlled by a global regulatory network in Gram-negative bacteria (Kertesz 2000), and an understanding of the monooxygenase system involved in desulfonating arylsulfonates is becoming available at the molecular level (Kertesz and Kahnert 2001). The system is widespread in soils (Lechner and Cook, unpublished). Analogous regulatory and desulfonative systems are presumably present in Gram-positive bacteria (van der Ploeg *et al.* 1998); coryneforms, especially *Rhodococcus* spp., are well known in desulfurisation and desulfonation (Omori *et al.* 1992; Omori *et al.* 1995; Chien *et al.* 1999).

This phenomenon of desulfonation of apparently recalcitrant compounds has been observed several times (Zürrer *et al.* 1987; Kertesz *et al.* 1994; King and Quinn 1997; Key *et al.* 1998; Mampel *et al.* 1998; Rein and Cook 1999; Ruff *et al.* 1999; see also Denger and Cook

1999). Indeed, the failure to obtain an enrichment, seen above with LADPEDS, is unusual in our experience (Cook, unpublished). The desulfonation products, in contrast to the sulfonates, are reactive compounds which can bind to soils, polymerise or be subject to biodegradation (Rein and Cook 1999; Ruff *et al.* 1999). The rationale is that the conversion of an arylsulfonate to a phenol will make the latter much more reactive and liable to attack than the former (Wellens 1990).

We believe that *P. lavamentivorans*^{T} DS-1 is representative of organisms in many pristine and contaminated environments (Schleheck *et al.* 2000; Dong, Knepper and Cook, unpublished). In sewage works, with their high levels of sulfate ions, LADPEDS will only be converted to DSDPEC (Fig. 1). In soil, with its sulfate limitation (Kertesz 2000), the DSDPEC formed by organisms like strain DS-1^T will presumably be subject to desulfonation by organisms like *R. opacus* ISO-5. The resulting MSDPEC-phenol and DPEC-diphenol are then available for dissimilation, as indicated above. We feel this to be a rational explanation of the observations in the industrial lab (see Introduction) (Quencer and Loughner 2001).

We have been unable to quantify many of the reactions we have observed, because of the complexity of the substrates involved in the various reactions, and because of the lack of authentic standards. Quantification may be possible if a single species of LADPEDS were available, which should allow quantitative identification of the DSDPEC species formed by strain DS-1^T. Individual compounds, in turn, could be supplied to strain ISO-5 to follow desulfonation. And work with single compounds would allow further desulfonation or dissimilation to be studied in detail.

ACKNOWLEDGMENTS

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CHAPTER 5

Mineralization of individual linear alkylbenzenesulfonate (LAS) congeners (2-C10-LAS, 2-C11-LAS, and 3-C12-LAS) by defined pairs of heterotrophic bacteria

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ABSTRACT

Parvibaculum lavamentivorans^{T} DS-1 utilises commercial linear alkylbenzenesulfonate (LAS) quantitatively by attacking and shortening the sidechain: about 9 major sulfophenyl-carboxylates (SPCs) and many similar compounds (e.g. α,β-unsaturated SPCs, SPC-2H) were excreted. 2-(4-Sulfophenyl)decane (2-C10-LAS) was converted largely to 3-(4-sulfophenyl) butyrate (3-C4-SPC), as were 2-C12-LAS and 2-C14-LAS. 2-C11-LAS was converted largely to 4-C5-SPC and we confirmed that 3-C12-LAS was converted largely to 4-C6-SPC. Traces of many other SPCs were found (up to 20 % of the carbon), and the patterns of identified products confirmed that LAS is degraded by ω-oxygenation and chain-shortening through β-oxidation. We isolated *Comamonas testosteroni* strains SPB-2 and KF-1, which utilised 3-C4-SPC. Another new isolate, *Delftia acidovorans* SPH-1, utilised 4-C6-SPC enantioselectively. Substrate-dependent oxygen uptake of whole cells of strain SPB-2 indicated inducible oxygenation of 3-C4-SPC and of 4-sulfophenol in whole cells of the strains of *C. testosteroni* during growth with 3-C4-SPC or 4-sulfophenol. The degradative pathways apparently involved 4-sulfocatechol and 4-sulfocatechol-1,2-dioxygenase. Strain SPB-2 and strain DS-1^{T} grew together in LAS-salts medium: only 7 major SPCs were recovered, so strain SPB-2 utilised 3-C4-SPC, 3-C5-SPC, and the minor product, 3-C4-SPC-2H. Strain SPH-1 also grew together with strain DS-1^{T} in LAS-salts medium: again only 7 major SPCs were recovered, so strain SPH-1 utilised 4-C6-SPC, 4-C5-SPC, and the minor products, 4-C6-SPC-2H and 4-C5-SPC-2H. The three-member community of strains DS-1^{T}, SPB-2 and SPH-1 utilised four major SPCs (and the minor products). We infer that this community mineralises the major SPCs derived from eight of the 20 LAS congeners, and that many more organisms are needed to complete a community able to mineralise all SPC-like compounds from commercial LAS.

INTRODUCTION

Linear alkylbenzenesulfonate (LAS) was proven to be biodegradable in 1957 (Sawyer and Ryckman 1957) and it is the major synthetic surfactant in world wide use (e.g. Schulze 1996). One can calculate that LAS represents some 4 % of the organic input into sewage treatment plants in western Europe (http://www.lasinfo.org), where it is presumably the highest bulk compound to be degraded. Nonetheless, a complete degradative pathway for LAS, backed by scientific evidence, is only now becoming available. It was many years after the requirement for communities to degrade the commercial product was recognised (Jiménez *et al.* 1991; Sigoillot and Nguyen 1992; Hrsák 1995a), at which time routine analytical-chemical methods for the microbiology laboratory were largely unavailable (Matthijs and De Henau 1987; Kölbener *et al.* 1995b; Eichhorn and Knepper 2002), and the lack of pure cultures (Hrsák and Begonja 2000; Schleheck *et al.* 2000; Schulz *et al.* 2000; Schleheck *et al.* 2003e) prevented advances. The first suggestions on community structure were, however, being made (van Ginkel 1996; Hrsák and Begonja 1998; Cook and Hrsák 2000; Dong *et al.* 2003).

Commercial LAS is ideally a mixture of 20 congeners (Fig. 1), which are degraded to a mixture of sulfophenylcarboxylates (SPC), sulfophenyldicarboxylates (SPdC) and α,β-unsaturated sulfophenylcarboxylates (SPC-2H) by *Parvibaculum lavamentivorans*^{T} DS-1 in a pattern that confirms the degradative pathway to involve ω-oxygenation and β-oxidation (Dong *et al.* 2003; Schleheck *et al.* 2003e), as first illustrated in mixed cultures by Eichhorn and Knepper (Eichhorn and Knepper 2002). This mixture of products is termed SP(d)C.

Strain DS-1^{T} generates mainly 4-(4-sulfophenyl)hexanoate (4-C6-SPC) from 3-(4-sulfophenyl)dodecane (3-C12-LAS) (Schleheck *et al.* 2000; Dong *et al.* 2003). Cook and Hrsák (Cook and Hrsák 2000) deduced a three-tier model for the degradation of LAS initiated by

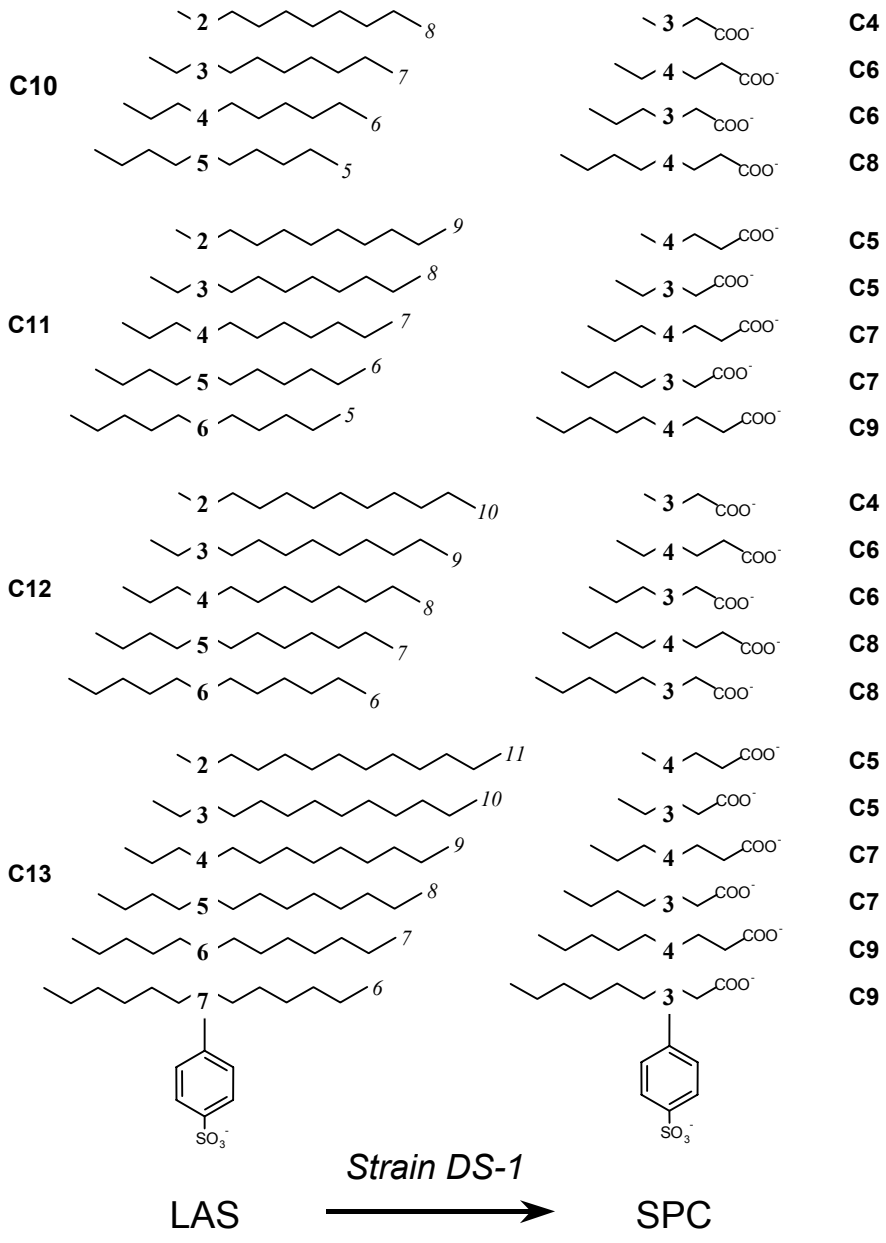

FIG. 1. Diagramatic representation of the 20 congeners of commercial LAS and of the corresponding major SPC known or presumed to be formed from each congener by *P. lavamentivorans*^{T} DS-1.

methanotrophic bacteria and completed by heterotrophic bacteria, and for purely heterotrophic degradation in which SPdCs played an important role. The model is still valid for the defined methanotrophic community, but we were working with a very limited range of organisms (e.g. *Delftia acidovorans* SPB1) able to mineralise only one SPC, 2-(4-sulfophenyl)butyrate (2-C4-SPC). 2-C4-SPC is degraded *via* 4-sulfocatechol and *ortho* ring cleavage with subsequent desulfonation (Schulz *et al.* 2000).

We now report that a two-tier, heterotrophic, bacterial community can be found for each of three LAS congeners which we believe to represent 8 of the 20 congeners of LAS. The first organism in each community is *P. lavamentivorans*^{T} DS-1 (Schleheck *et al.* 2000; Schleheck *et al.* 2003e), which yields one major product, and several minor products, from each LAS congener. The second organism degrades this major SPC, the corresponding SPC-2H, and a second major SPC, *via* oxygenation of the SPC and *ortho* cleavage of 4-sulfocatechol. We conclude that a large community is needed to degrade LAS completely.

MATERIAL AND METHODS

Materials

Commercial LAS (C10 to C13) with a mean sidechain of 11.4 carbon atoms (Kölbener *et al.* 1995a), Marlon A350, was provided by Hüls (Marl, Germany). The single LAS-congeners 2-C10-LAS, 2-C12-LAS and 2-C14-LAS were gifts from Petresa (Madrid, Spain) (each of > 98 % purity), and 3-C12-LAS was made available by CONDEA-Vista (Austin, USA) (about 95 % pure; see ref. Dong *et al.* 2003). Commercial LAS and the single LAS congeners were prepared in 10 mM stock solutions in destilled water and diluted into minimal-salts medium to a final concentration of 1 mM; 10 mM 2-C14-LAS precipitated at room temperature and was re-dissolved by heating to over 60 °C (microwave oven) prior to dilution. Racemic 2-(4-sulfophenyl)butyrate (2-C4-SPC) was available in the laboratory (Schulz *et al.* 2000), and racemic 3-C4-SPC was synthesised by reacting sulfuric acid with the corresponding phenylcarboxylic acid followed by re-crystallisation as described by Schulz et al. for synthesis of 2-C4-SPC (Schulz *et al.* 2000). The compound was obtained in about 95 % purity (major by-product 2-(2-

sulfophenyl)butyrate) and its identity was confirmed by electrospray mass spectroscopy and nuclear magnetic resonance (cf. Schulz *et al.* 2000).

4-Sulfocatechol was kindly supplied at about 95 % purity by B. Feigel (Feigel and Knackmuss 1993); the major impurity was 3-sulfocatechol. Agarose (ultra-pure electrophoresis grade; FMC) was used to solidify SPC-salts medium for selective plating. The starting materials for chemical synthesis were purchased from Fluka (Buchs, Switzerland), and the sources of routine chemicals were given elsewhere (Kölbener *et al.* 1994; Schulz *et al.* 2000).

Analytical methods

LAS and SPC were routinely analysed by gradient, reversed-phase HPLC coupled to a UV (diode-array) detector with gradient system I (Schleheck *et al.* 2000). HPLC for mass spectroscopy (gradient system II) and determination of LAS and SPC via an electrospray interface coupled to a mass spectrometer (ES-MS) operating in the negative-ion mode was described by Eichhorn et al. (Eichhorn and Knepper 2002). LAS and SPCs were tentatively identified by *m/z*-value of the deprotonated molecular ions, and the identity was confirmed when fragments with *m/z* = 183 (4-styrenesulfonate) and *m/z* = 119 (4-styrenephenolate) was observed (Eichhorn and Knepper 2002). SPC-2Hs were detected by an *m/z*-value of the deprotonated molecular ions 2 units lower than that of the corresponding SPC, and confirmed when the characteristic fragment pattern was observed (Eichhorn and Knepper 2002).

The separation of SPCs with gradient system II was less efficient than that with gradient system I (UV-detector). This was not a problem for HPLC-ES-MS (gradient system II), where individual ions were traced, but it did present a problem comparing data between the routine separations in Konstanz (gradient system I, shown in this paper), where the biological experiments were done, and the corresponding detailed analyses and identifications in the LC-MS laboratory in Wiesbaden (see a comparison in Dong *et al.* 2003). However, the most important comparisons were made in the experiments in which the SP(d)Cs derived from individual congeners of LAS were subject to degradation in pure culture; here the chromatograms were simple enough, and the relative amounts of different compounds sufficiently different, to allow robust identifications of peaks to be made.

HPLC for enantioselective separation of *R*-4-C6-SPC and *S*-4-C6-SPC (gradient system III) was done with a method adapted from Schulz et. al (Schulz *et al.* 2000): 100-µl samples were loaded on to a β-pm-Nucleodex column (200 mm x 4 mm in diameter, 5 µm particle size, Macherey Nagel, Düren, Germany), that was equilibrated with 100 mM potassium phosphate buffer, pH 6.0, and after 5 min, a linear gradient to 50 % MeOH over 15 min was applied and maintained for 5 min. The *R*- and *S*-enantiomers eluted after 19.1 and 19.9 min, respectively, assuming that the same *R*-enantiomer of a compound always elutes first (Schulz *et al.* 2000).

The total LAS and SPC concentration in solutions was estimated photometrically as A 220 nm using pure compounds (2-C12-LAS or 3-C4-SPC) as standard. Growth was followed as turbidity (A_{580}) and quantified as protein in a Lowry-type reaction (Kennedy and Fewson 1968). Sulfate was determined

turbidimetrically as a suspension of $BaSO_4$ (Sörbo 1987). Substrate-dependent oxygen uptake of washed whole cells (or of cell extracts) and concomitant substrate degradation was determined as described elsewhere (Schleheck and Cook 2003a). Anoxic cell suspensions were prepared as described elsewhere (Schleheck and Cook 2003a).

Purification of 2-C11-LAS

2-C11-LAS was purified from commercial LAS by semi-preparative HPLC with a method adapted from HPLC gradient system I (see above). A semi-preparative C18-HPLC column (Ultraprep C18, 150 mm x 21.2 mm, 10 µm particle size, Beckman, USA) and a preparative HPLC system (Beckman System Gold, Preparative µ-Flow™) was used in combination with an fraction collector running automated in peak-cutting mode (Pharmacia LKB FRAC-100); LAS was detected at 260 nm. The flow rate was 5 ml/min, and the column was equilibrated with 65 % 0.11 M sodium perchlorate (buffer A) and 35 % acetonitrile. 5-ml Portions of 1 mM commercial LAS (acidified to pH 2 with 1 M HCl) were injected in each run, and after 5 min, the acetonitrile gradient was ramped to 70 % in 5 min, to 75 % in 10 min, and maintained at 75 % for 10 min; LAS eluted as sets of peaks from minute 18 to 36; 2-C11-LAS eluted as a single peak (at 26.5 min; Fig. 2A), which was collected. The initial conditions were restored within 5 min, and the column was re-equilibrated for 10 min prior to next injection. Samples of 2-C11-LAS from 20 separations was pooled, the volume reduced to about one third in a rotary evaporator at 60 °C (removal of acetonitrile), and diluted in the same volume of destilled water prior to adjustment of pH of 2 with HCl (1 M). This partially-purified 2-C11-LAS underwent a second purification (as above) to reduce impurities of, largely, 3- and 4-C11-LAS. These fractions were pooled, and acetonitrile was removed as above. The material was desalted by solid-phase extraction as described elsewhere (Schleheck *et al.* 2003d). The concentration of 2-C11-LAS was determined photometrically (220 nm) with 2-C10-LAS as standard.

Growth conditions for strain DS-1^T

A mineral-salts medium containing 50 mM potassium phosphate, pH 7.2, was used (Thurnheer *et al.* 1986), and strain DS-1^T was routinely grown with 1 mM commercial LAS (210 mg dissolved organic carbon/L) or single LAS congener(s) in buffered mineral-salts medium, that was supplemented by suspended glass particles (1 mg/ml) to allow growth of strain DS-1^T in presence of the surfactant (Schleheck *et al.* 2000; Schleheck *et al.* 2003e). The cultures were incubated at 30 °C in the dark, aerated on a roller when grown in the 3-ml scale (in 30 ml screw cap tubes), or on an orbital shaker when grown in the 250-ml scale (in 1-L Erlenmeyer flasks). When SPC-salts medium was to be generated, strain DS-1^T was grown with 2-C11-LAS, 2-C12-LAS or with 3-C12-LAS (or with commercial LAS), and complete conversion of LAS to SPC after growth was confirmed by HPLC (gradient system I). Bacteria and glass particles were collected by centrifugation (10,000 g, 60 min, 4 °C), and the supernatant fluid

was filtered (0.45 µm pore diameter) and autoclaved. This SPC-salts medium was used for carbon-limited enrichments of SPC-degrading bacteria (see below).

Enrichment and isolation of SPC-degrading bacteria

Enrichment cultures (3 ml) for bacteria able to utilise 3-C4-SPC or 4-C6-SPC as sole source for carbon and energy for growth were done in 1 mM-SPC-salts medium incubated at 30 °C on a rotary shaker in the dark; an untreated sample (30 µl) from the aeration tank of a sewage works was used as inoculum. The positive enrichments were subcultured several times into fresh selective medium. Bacteria were then plated on SPC-salts medium solidified with agarose (1 %), and on agarose-salts medium without additional carbon source (negative control), where pin-point colonies formed. A representative macrocolony from the SPC-salts medium was picked into the corresponding SPC-salts medium, and, after growth was observed, substrate utilisation was confirmed by HPLC (gradient system I). After several rounds of selective plating and picking, bacteria were plated on LB-medium (Gerhardt *et al.* 1994) to confirm the purity of the culture. The isolates were termed strain SPB-2 (from 3-C4-SPC medium) and strain SPH-1 (from 4-C6-SPC medium). Strain KF-1 was isolated essentially as described above, but the inoculum was biomass derived from a trickling filter able to mineralise commercial LAS (Dong *et al.* 2003), and the salts medium contained 6 mM 3-C4-SPC from the chemical synthesis. The identity of each isolate was determined under contract by the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) after sequencing a partial 16s rDNA-gene sequence, which was aligned and compared as described elsewhere (Rainey *et al.* 1996; Maidak *et al.* 2001).

Generation of growth curves

Samples were taken at intervals from 50-ml cultures; samples were used for HPLC-analyses, and for sulfate and for protein assays. Strain SPB-2, KF-1 or SPH-1 was grown in SPC-salts medium generated from LAS by strain DS-1^T (see above). Culture medium for growth of communities involving strain DS-1^T and SPC-degrader(s) in 1 mM LAS was supplemented with suspended glass particles (1 mg/ml; see Schleheck *et al.* 2000; Schleheck *et al.* 2003e).

RESULTS

Formation of SPCs from commercial LAS and from individual LAS-congeners during growth of strain DS-1^T

Strain DS-1^T grew with commercial LAS, which disappeared completely (Fig. 2A), to yield about 9 major and at least 15 minor peaks (Fig. 2B), which were shown to be SPCs, SPC-2Hs and SPdCs in earlier work (Schleheck *et al.* 2000; Dong *et al.* 2003). When a single congener of LAS (e.g. 3-C12-LAS) was used as the growth substrate, the congener disappeared, and a major product (4-C6-SPC) was formed, with several minor products (Fig. 3A, Table 1). The major product represented some 80 % of the total when 3-C12-LAS was used. The minor products attributable to 3-C12-LAS (Table 1) were 6-C8-SPC (about 10 %) and 4-C6-SPC-2H; the latter compound showed a UV-spectrum different to that of 4-C6-SPC (see below). Significant amounts of C5- and C7-SPC were also detected, but they were attributed to the 5 % impurities in the LAS (Eichhorn and Knepper 2002; Dong *et al.* 2003). The mass spectral identifications (Table 1) confirmed our earlier data (Dong *et al.* 2003). The molar growth yield from growth with 3-C12-LAS was 39 g protein/mol LAS, which confirmed our earlier data (Schleheck *et al.* 2000), represented about 6 mol C utilised/mol LAS (Cook 1987) and thus mass balance for the utilised carbon.

Strain DS-1^T, when supplied with 1 mM 2-C10-LAS, 1 mM 2-C12-LAS or 1 mM 2-C14-LAS in salts medium, generated 39, 47 or 61 µg protein/ml (each value the mean of three experiments) and yielded the same major SPC in each case, as illustrated in an experiment with 2-C12-LAS or all three congeners in growth medium (Fig. 3BC). This major product co-chromatographed with authentic 3-C4-SPC, had the same UV-spectrum as the authentic material, and the identification was confirmed by LC-ES-MS (Table 1). The growth yields were consistent

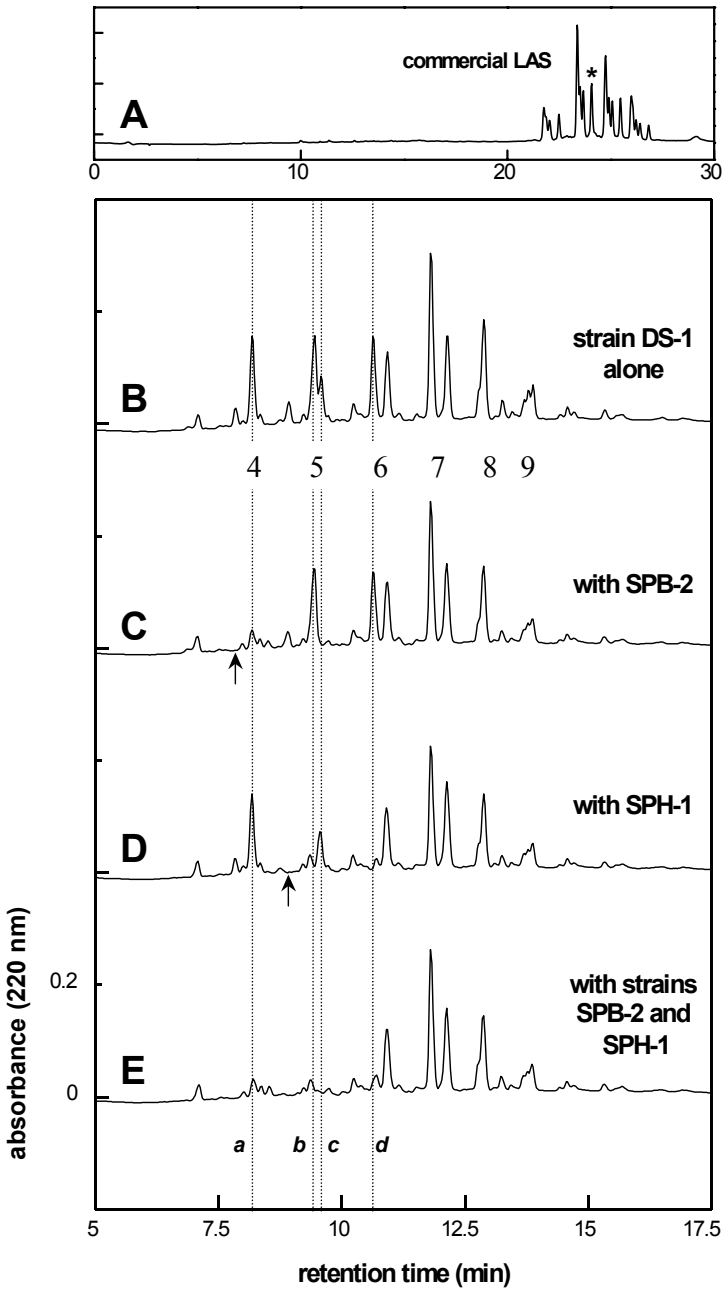

FIG. 2. HPLC chromatograms of commercial LAS in salts-medium (A), of the culture medium after growth of *P. lavamentivorans*^{T} DS-1 (B), after growth with strains DS-1^T and SPB-2 (C), after growth with strains DS-1^T and SPH-1 (D) and after growth with strains DS-1^T, SPB-2 and SPH-1 (E). The asterisk, *, marks 2-C11-LAS, which was isolated in this work. The numbers (4-9) in Fig. 2B represent the chain lengths for the major SPCs formed. The letters 'a-d' refer to peaks represent identified major SPCs which were subject to degradation, whereas the arrows indicate the minor products (SPC-2Hs) which were degraded (see text).

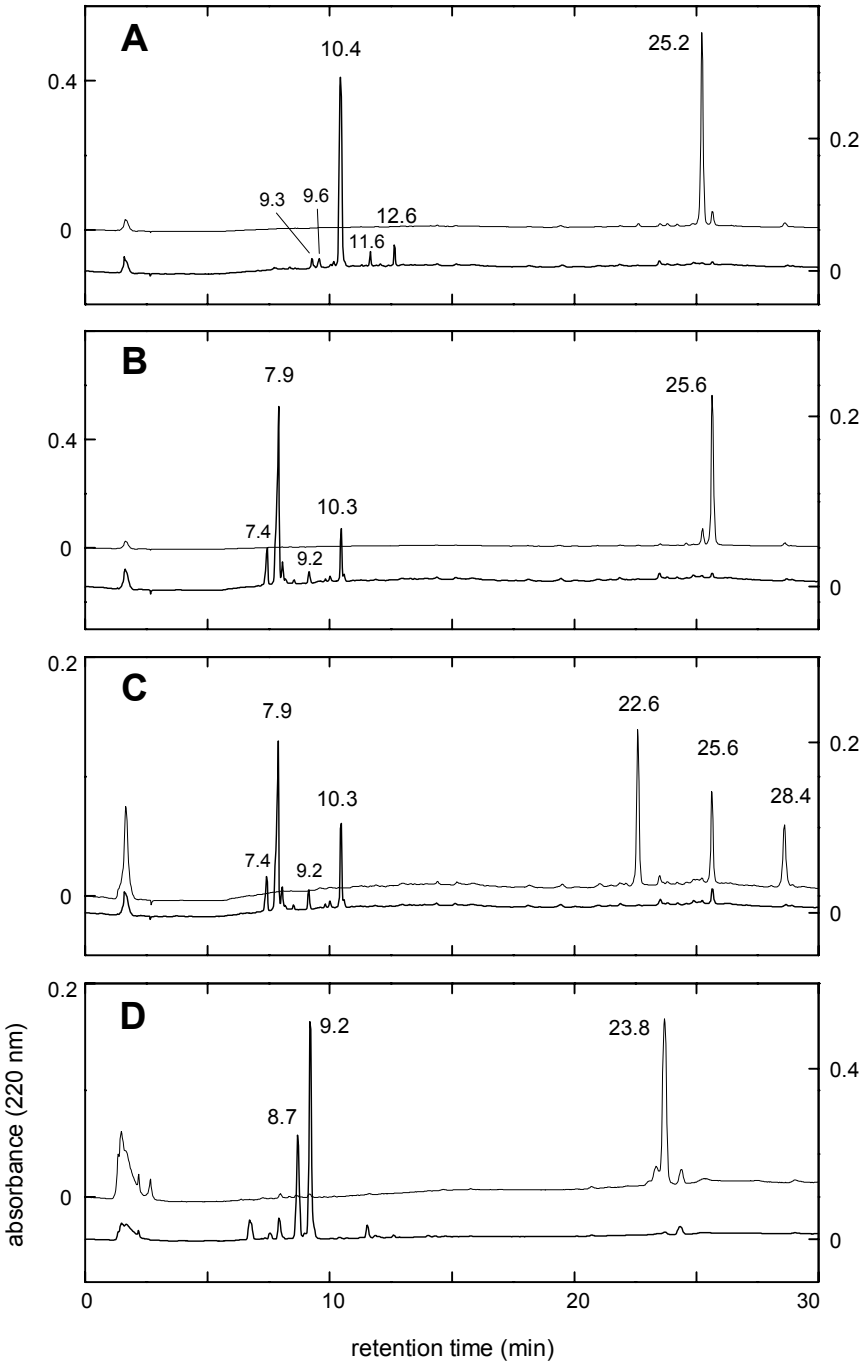

FIG. 3. HPLC chromatograms of one or more congeners of LAS in salts-medium (upper curve of each pair) and of the culture medium after growth of *P. lavamentivorans*^{T} DS-1 (lower curve of each pair). 1 mM 3-C12-LAS (A), 1 mM 2-C12-LAS (B), a mixture of 2-C10-LAS, 2-C12-LAS and 2-C14-LAS (each 0.33 mM) (C), and 0.5 mM 2-C11-LAS (D). Analyses were done with HPLC gradient system I.

TABLE 1. Retention times (gradient system I) of SPCs formed during growth of *P. lavamentivorans*[T] with single LAS congeners (cf. Fig. 4), contribution of the relevant peak to total SPC-peak area (A_{220}), the corresponding *m/z*-signals ([*M*-H]$^-$) observed by LC-ES-MS (gradient system II), the general characteristics of the SPC, and the presumed identity of the compound.

Growth substrate	Product				Interpretation [a]
	Retention time (*min*) [b]	Peak area (*%*) [c]	*m/z*	SPC [d]	
3-C12-LAS [g]	9.3	2	243	C5	3-C5-SPC [e]
	9.6 [f]	2	269	C6-2H	4-C6-SPC-2H
	10.4	85	271	C6	4-C6-SPC
	11.7	3	285	C7	5-C7-SPC [e]
	12.6	7	299	C8	6-C8-SPC
2-C12-LAS	7.4 [f]	13	241	C4-2H	3-C4-SPC-2H
	7.9	70	243	C4	3-C4-SPC
	9.2	2	257	C5	4-C5-SPC [h]
	10.3	12	271	C6	5-C6-SPC
2-C10-LAS 2-C12-LAS 2-C14-LAS	7.4 [f]	9	241	C4-2H	3-C4-SPC-2H
	7.9	62	243	C4	3-C4-SPC
	9.2	5	257	C5	4-C5-SPC [h]
	10.3	21	271	C6	5-C6-SPC
2-C11-LAS	8.7 [f]	31	255	C5-2H	4-C5-SPC-2H
	9.2	53	257	C5	4-C5-SPC

[a] Interpretation derived from LC-ES-MS-data, UV-spectrum and retention time, the substrate range of SPC-degrading isolates, and the idea that the substituent does not migrate on the chain.

[b] Only peaks clearly attributable as products from degradation of the relevant LAS congener(s) are given.

[c] Percent of total SPC-peak area.

[d] Derived from the LC-ES-MS signal.

[e] This material probably arose largely from contaminants in the LAS.

[f] This material showed an anomalous UV-spectrum (see Fig. 5).

[g] These data represent an evaluation of a new version of an experiment shown by Dong et al. (2003).

[h] This product was presumably formed after an α-oxidation step (see Discussion).

with this identification, because they indicated utilisation of 6, >7 and >9 carbon atoms from the C10-LAS, the C12-LAS and the C14-LAS, respectively.

The major product from these three congeners was 3-C4-SPC, but as with 3-C12-LAS, there were several other products (Fig. 3BC). The peak at 10.3 min was a C6-SPC as identified by LC-ES-MS, and it could be separated from 4-C6-SPC (Table 1) (i.e. was not utilised by 4-C6-SPC-degrading isolate *D. acidovorans* SPH-1, see below), thus was presumably 5-C6-SPC (Fig. 4), and it represented about 20 % of the relevant products when the congeners 2-C10-LAS, 2-C12-LAS or 2-C14-LAS were used as single growth substrates (Table 1). The peak at 7.4 min (Fig. 3BC) was shown to be an α,β-unsaturated C4-SPC by LC-ES-MS, and was presumably 3-C4-SPC-2H (Fig. 2). The peak at 9.2 min (Fig. 3BC) was a C5-SPC as shown by LC-ES-MS: this compound co-eluted with 4-C5-SPC (see below) (i.e. was utilised by 4-C5-SPC-degrading *D. acidovorans* SPH-1, see below). Impurities in the LAS were negligible, especially when the mixture of 2-C10-, 2-C12-, and 2-C14-LAS was used, and we presume that this 4-C5-SPC is a minor product generated from 2-C10-, 2-C12-, and 2-C14-LAS. This is the first evidence for a trace of α-oxidation in *P. lavamentivorans*^{T}.

2-C11-LAS from commercial LAS was available at about 80 % purity (major impurities 3- and 4-C11-LAS, and 6-C12-LAS). Strain DS-1^T converted this material during growth to a major product (Fig 3D; peak at 9.2 min) identified as a C5-SPC as by LC-ES-MS (Table 1), which we presume to be 4-C5-SPC (Fig. 1). The product with a retention time of 8.7 min (Fig. 3D) was an α,β-unsaturated C5-SPC (Table 1), presumably 4-C5-SPC-2H. Minor products observed in this experiment (Fig. 3D) were a C7-SPC (putative 6-C7-SPC) (11.6 min), which was presumably formed from 2-C11-LAS, and a C4-SPC (7.9 min) of unknown origin. The peak at 6.9 min (Fig. 3D) eluted too early from the corresponding separation by LC-ES-MS to yield an identification.

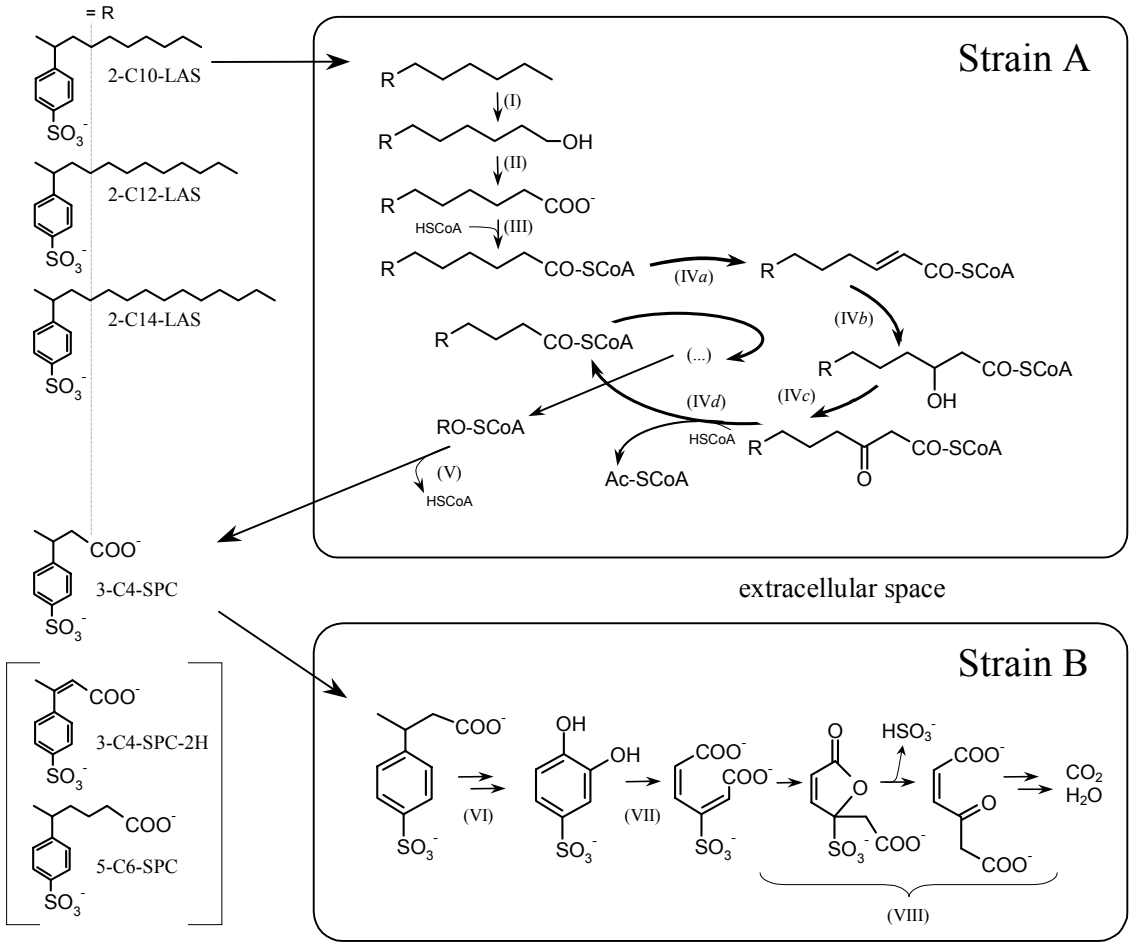

FIG. 4. Sketch of the conversion of LAS (here 2-C10-LAS, 2-C12-LAS and 2-C14-LAS) to SPCs and complete degradation of the major SPC (3-C4-SPC) by a two-tier bacterial community. Strain A is represented by *P. lavamentivorans*^{T} DS-1, and strain B by *C. testosteroni* SPB-2, in this work. Degradation is initiated by ω-oxygenation and oxidation of LAS to the corresponding SPC (Dong *et al*. 2003), which is considered to undergo β-oxidation until hindered by the 4-sulfophenyl-substituent; the unsubstituted SPC is then excreted. The SPC is apparently oxygenated, possibly via 4-sulfophenol, to 4-sulfocatechol, which is subject to *ortho*-cleaved and degradation. Reactions catalyzed by *P. lavamentivorans*^{T} DS-1 after uptake of LAS: (I), ω-oxygenation; (II), ω-oxidations; (III), thioesterification; (IV*a*-IV*d*), spirals of β-oxidation contributed by α,β-desaturation, addition of water, (true) β-oxidation, and β-ketothiolysis; (V) thioester cleavage and excretion. Reactions catalyzed by *C. testosteroni* SPB-2 after uptake of 3-C4-SPC: (VI) initial oxygenation(s) of 3-C4-SPC and side-chain removal; (VII), 4-sulfocatechol *ortho*-cleavage, (VIII), modified *ortho*-degradation pathway and central metabolism.

The UV-spectra of the α,β-unsaturated SPCs

Previously, we reported that the UV-spectra of products from LAS resembled that of e.g. 3-C4-SPC (Fig. 5) (Schleheck *et al.* 2000), and the identified SPCs in this work confirmed this (not shown). However, with access to separated, low-quantity products, we observed that 3-C4-SPC-2H has a different UV-spectrum (Fig. 5). The maxima and minima at higher wavelengths were consistent with a larger system of delocalized electrons, analogous to the delocalization in 4-sulfostyrene and its UV-spectrum (Fig. 5). The UV-spectrum of 4-C5-SPC-2H (maxima at 204 and 264 nm, minimum at 237 nm) was identical to that of 3-C4-SPC-2H (Fig. 5).

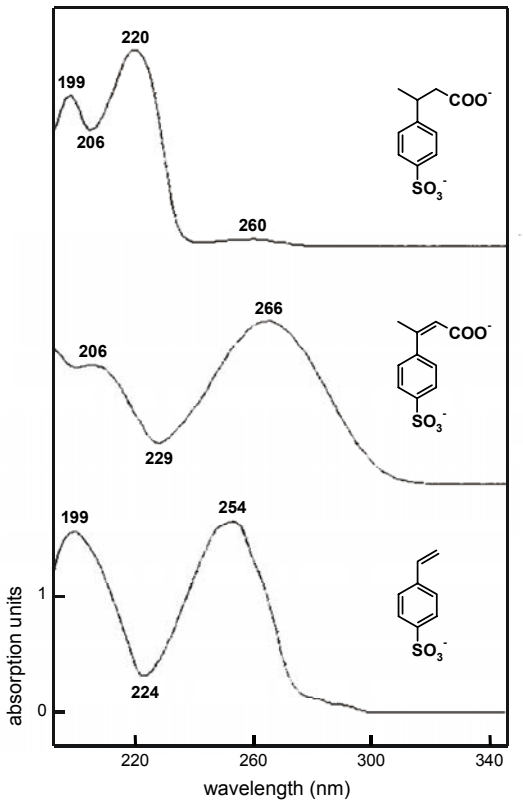

FIG. 5. UV-spectra of 3-C4-SPC, 3-C4-SPC-2H and 4-styrenesulfonate obtained separated in gradient system I. 3-C4-SPC and 3-C4-SPC-2H were generated from 1 mM 2-C12-LAS, and the spectra obtained from separations of ten-fold or undiluted samples, respectively. The spectrum of 4-styrenesulfonate was obtained from the separation of a sample containing 60 μM 4-styrenesulfonate.

Enrichment cultures for SPC-degrading bacteria

We enriched from communal sewage sludge bacteria that were able to grow with 4-C6-SPC (major SPC from 3-C12-LAS) or with 3-C4-SPC (major SPC from 2-C12-LAS) as sole source for carbon and energy. Growth of bacteria (mostly rods) in each enrichment culture was observed after three days, and no bacteria grew in the negative control (no carbon source). After several transfers into fresh medium, the cultures were plated on 4-C6-SPC (or 3-C4-SPC) medium solidified with agarose; agarose salts medium without added carbon source was used as the negative control. Growth to large (3 mm diameter) colonies of bacteria was observed on 4-C6-SPC (or 3-C4-SPC) plates, whereas pin-point colonies were found on the negative controls. Only the large colonies (rods) grew in selective liquid medium, where we observed growth and substrate disappearance (HPLC). After several rounds of selective plating and picking, the culture was streaked on complex medium, where homogenous colony morphology was observed. Bacteria grew when transferred to selective medium and they utilised the substrate.

The isolate which utilised 4-C6-SPC was termed strain SPH-1. It was a Gram-negative, motile rod. The partial 16S-rDNA sequence showed 100 % sequence identity with that of *Delftia acidovorans*^{T} (DSM 39). *Delftia acidovorans* SPH-1 was deposited with the DSMZ as DSM 14801.

The isolate which utilised 3-C4-SPC was termed strain SPB-2. It was a Gram-negative, motile rod. The partial 16S-rDNA sequence showed 100 % sequence identity with that of *Comamonas testosteroni*^{T} (ATCC 11996). *Comamonas testosteroni* SPB-2 was deposited in the DSMZ as DSM 14802.

We obtained a second bacterium, strain KF-1, able to utilise 3-C4-SPC. This was isolated from a trickling filter which degraded LAS quantitatively (Dong *et al.* 2003). The organism was a Gram-negative, motile rod whose partial 16S-rDNA sequence showed 100 % sequence identity with that of *Comamonas testosteroni*^{T} (ATCC 11996). *Comamonas testosteroni* KF-1 was

deposited with the DSMZ as DSM 14576. *C. testosteroni* strains KF-1 and SPB-2 showed different colony morphology when grown on LB-plates (vaulted-dense and spread-diffuse colonies, respectively).

Growth of SPC-degrading bacteria

D. acidovorans SPH-1 was grown in minimal-salts medium, which contained 4-C6-SPC (and other SPCs) generated from 3-C12-LAS (Table 1). Comparison of the medium before and after growth (Fig. 6A) showed that only 4-C6-SPC and one minor product from LAS, 4-C6-SPC-2H, were degraded. 6-C8-SPC (Table 1) and other minor products were not utilised. Corresponding to this observation, the residual level of dissolved organic carbon in the culture medium was about 20 % of the initial, consistent with the residual peak area. When strain SPH-1 was incubated in the minimal-salts medium containing 3-C4-SPC (and other SPCs) generated from 2-C12-LAS (Table 1), only 4-C5-SPC disappeared (the product presumed from α-oxidation, see above), but 3-C4-SPC, 3-C4-SPC-2H, and 5-C6-SPC were not utilised; 2-C4-SPC was also not utilised when tested. The utilisation of 4-C5-SPC and of 4-C5-SPC-2H by strain SPH-1 was confirmed by analysis of the single growth experiment possible in the small amount of 4-C5-SPC medium, which was generated from purified 2-C11-LAS (Fig. 4D): no other assays were possible. The organism utilised benzoate, phenylacetate, 2- and 3-hydroxyphenylacetate, 4-hydroxybenzoate, 3,4-dihydroxybenzoate (protocatechuate), 4-phenylbutyrate, and 4-sulfocatechol as sole carbon sources for growth, but not 4-sulfophenol, 4-sulfobenzoate, 3-phenylbutyrate or 4-sulfostyrene.

The *R*- and *S*-enantiomers of 4-C6-SPC were subject to separation (Fig. 7, inset) (gradient system III). Strain SPH-1 initially utilised solely the putative *S*-enantiomer (Fig. 7), then both enantiomers till the *S*-enantiomer was exhausted, and finally the residual *R*-enantiomer alone. The molar growth yield of 5.3 g protein/mol carbon indicated quantitative substrate utilisation (Cook 1987). The sulfonate sulfur was released concomitantly with growth and was

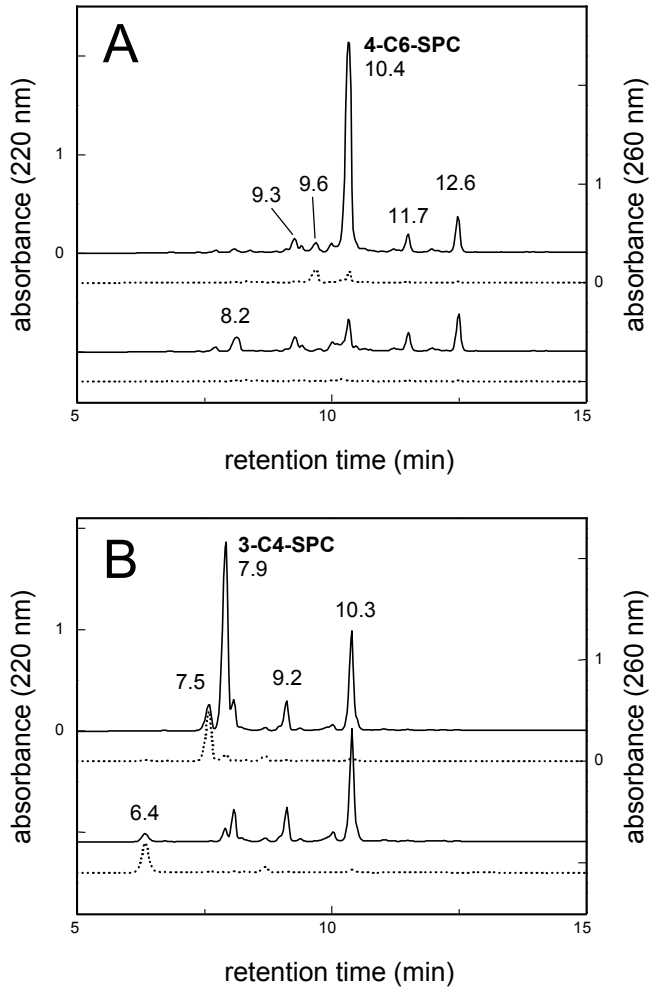

FIG. 6. HPLC chromatograms (gradient system I) of culture medium before (upper sets) and after (lower sets) growth of *D. acidovorans* SPH-1 with 4-C6-SPC (A) and of *C. testosteroni* SPB-2 with 3-C4-SPC (B). The detector was operated at 220 nm (solid line) and 260 nm (dots) to distinguish peaks with different absorption characteristic (SPC and SPC-2H).

recovered, essentially quantitatively, as sulfate (Fig. 7). There was thus essentially a complete mass balance for the utilisation of 4-C6-SPC, so a minor product (retention time 8.2 in Fig. 6A, and with the UV-spectrum of an SPC), which accumulated concomitant with growth contained an insignificant amount of carbon.

C. testosteroni SPB-2 (or *C. testosteroni* KF-1, not shown) was grown in the minimal-salts medium containing 3-C4-SPC (and other SPCs) generated from 2-C12-LAS (Table 1). Comparison of the medium before and after growth (Fig. 6B) showed that only 3-C4-SPC and 3-

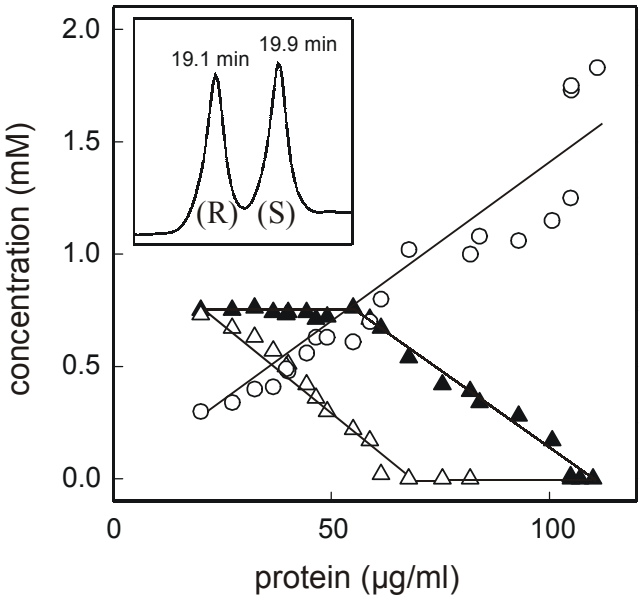

FIG. 7. Differential plot (concentration *vs.* protein) of growth of *D. acidovorans* SPH-1 utilising 4-C6-SPC in salts medium, with, inset, the enantiomeric separation of *R*- and *S*-4-C6-SPC. Symbols: Δ, putative *S*-4-C6-SPC; ▲, putative *R*-4-C6-SPC; ○, sulfate.

C4-SPC-2H were degraded. 5-C6-SPC and other minor products were not utilised. The residual dissolved organic carbon, about 25 % of the initial value, corresponded to this observation. When strain SPB-2 or KF-1 was incubated in the minimal-salts medium, which contained 4-C6-SPC (and other SPCs) generated from 3-C12-LAS (Table 1), 3-C5-SPC disappeared, but 4-C6-SPC, 4-C6-SPC-2H, and 6-C8-SPC were not utilised (not shown); 2-C4-SPC was also not utilised. Each organism utilised *p*-sulfophenol, benzoate, 4-hydroxybenzoate, 3,4-dihydroxybenzoate, 2- and 3-hydroxyphenylacetate as sole sources of carbon and energy for growth. The organisms did not utilise 4-sulfobenzoate, phenylacetate, 3- and 4-phenylbutyrate or 4-sulfostyrene; strain SPB-2 utilised 3-phenylpropionate, whereas strain KF-1 did not.

We could not separate the enantiomers of 3-C4-SPC. During exponential growth of strain SPB-2 ($\mu = 0.22\ h^{-1}$; not shown) in 3-C4-SPC medium derived from 2-C10-LAS, the utilisation of 3-C4-SPC (and of 3-C4-SPC-2H) was concomitant with growth and with the excretion of sulfate. Recovery of sulfate (83 % of total sulfonate) and a molar growth yield of 6.1 g protein

per mol of carbon utilised indicated quantitative substrate utilisation, and mass balance. The metabolic product, which eluted at 6.3 min (Fig. 6B), thus represented a negligible amount of carbon.

Tolerance of strains SPH-1, SPB-2 and KF-1 to LAS

The new isolates grew without clumping in suspended culture with e.g. succinate as the sole source of carbon and energy. The organisms could grow in presence of LAS at concentrations up to 0.5 mM LAS, but growth was now accompanied by formation of a biofilm. *D. acidovorans* SPH-1 tended to wall growth under these conditions whereas *C. testosteroni* strains SPB-2 and KF-1 tended to form clumps. The addition of glass particles (1 mg/ml) to the culture medium containing LAS allowed the organisms to grow with a shorter lag-phase: clumping of the glass particles during growth was due to the development of biofilm on the solid support (not shown). *C. testosteroni* KF-1 grew in a uniform suspension in 3-C4-SPC-salts medium, whereas strain SPB-2 tended to form clumps, so we largely used strain KF-1 for experiments with whole-cell suspensions (see below).

Mineralization of 3-C12-LAS or 2-C12-LAS in defined two-member communities

P. lavamentivorans^{T} DS-1 utilised 3-C12-LAS as the sole added growth substrate in a single growth phase and excreted largely 4-C6-SPC (and minor SPCs) with utilisation of some sulfate (Fig. 8A); the organism grew in a biofilm on glass particles from which it could be shaken loose for a turbidity measurement, as described previously (Schleheck *et al.* 2000; Schleheck *et al.* 2003e). In the additional presence of *D. acidovorans* SPH-1, a two-phase growth curve was observed (Fig. 8B). In the first phase, LAS was utilised and 4-C6-SPC was excreted, but negligible turnover of the latter occurred, as shown by the negligible release of sulfate in the presence of LAS, and by the quantitative release of the SPC (at about 3 days). The SPC was utilised quantitatively in the second phase of growth, which appears to be uncoupled

from substrate disappearance (during day 3) but simultaneous with release of sulfate (day 4). This was probably due to extreme clumping in the culture, which was not detected as turbidity till the clumps disintegrated and presumably released sulfate. We could not determine growth directly as protein, because there was insufficient 3-C12-LAS to allow an experiment of that magnitude. For the same reason, we could not follow the behaviour of the enantiomers of the SPC (cf. Fig. 7).

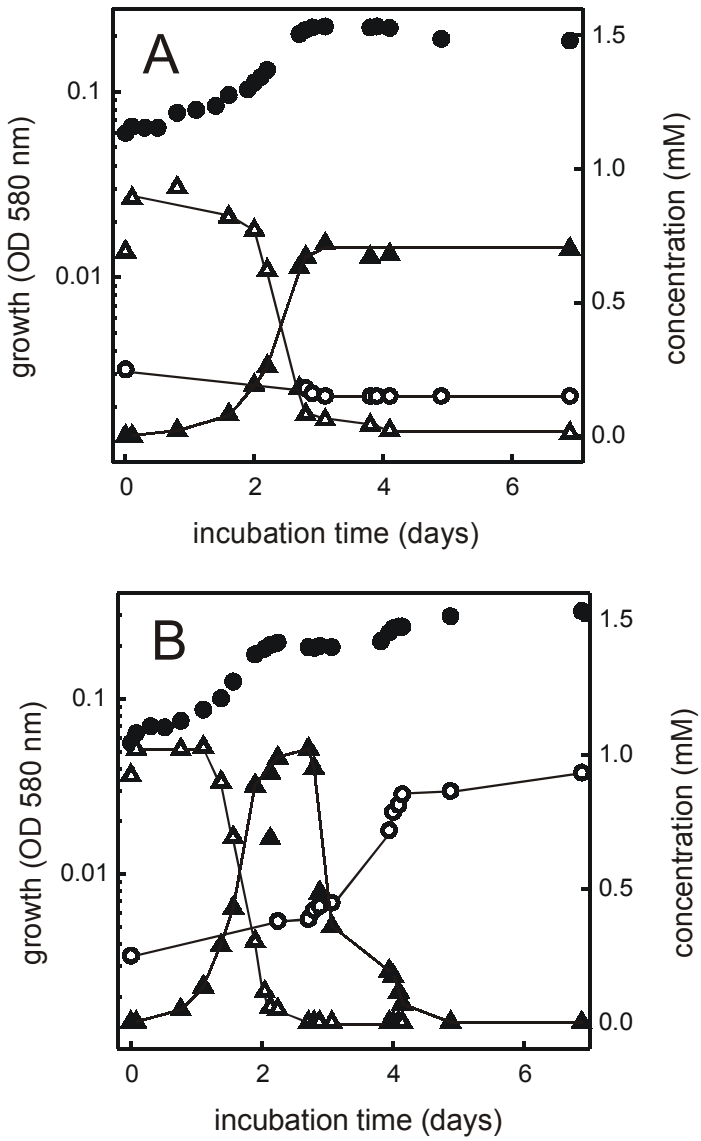

FIG. 8. Growth of *P. lavamentivorans*^{T} DS-1 (A) and of the community of strain DS-1^T with *D. acidovorans* SPH-1 (B) in 3-C12-LAS-salts medium. Symbols: ●, turbidity; Δ, 3-C12-LAS; ▲, 4-C6-SPC; ○, sulfate.

Similar experiments were done with 2-C12-LAS, *P. lavamentivorans*^{T} DS-1 and *C. testosteroni* SPB-2. The data from the community also showed two-phase growth with quantitative utilisation of both LAS and the corresponding 3-C4-SPC, and the quantitative release of sulfate (data not shown).

Mineralization of several congeners of LAS in two- and three-member communities

Strain DS-1^T was grown in salts-medium containing 1 mM commercial LAS alone to yield a mixture of SP(d)Cs (Fig. 2B), or in the presence of *C. testosteroni* SPB-2 (Fig. 2C), *D. acidovorans* SPH-1 (Fig. 2D), or both (Fig. 2E). The initial experiments (see above) indicate that strains SPB-2 and SPH-1 have narrow substrate ranges for SPCs, though not as narrow as the single SPC-substrate known for the older isolate, *D. acidovorans* SPB1 (Schulz *et al.* 2000). Strain SPB-2 utilised only 3-C4-SPC (peak 'a' in Fig. 2), 3-C4-SPC-2H (arrow in Fig. 2B), and 3-C5-SPC (peak 'c' in Fig. 2). Strain SPH-1 probably utilised four compounds, 4-C6-SPC (peak 'd' in Fig. 2), 4-C5-SPC (peak 'b' in Fig. 2) and 4-C5-SPC-2H (arrow in Fig. 2C); 4-C6-SPC-2H, which was also subject to degradation (see Fig. 6A), was not detected in Fig. 2. The three-member community degraded precisely the same six SPCs (a-d, arrows) (Fig. 2E), so no interference amongst community members was detected.

Oxygenase activities detected in SPC-degrading organisms

Whole cells of *D. acidovorans* SPH-1 showed 4-sulfocatechol-dependent oxygen uptake (0.2 mkat/kg protein) when grown with 4-C6-SPC (Table 2). No development of yellow colour, which might indicate *meta*-ring cleavage, was detected, and the results were interpreted as a preliminary evidence for *ortho*-ring cleavage of 4-sulfocatechol. The very limited amounts of biomass from strain SPH-1 available under these conditions precluded further direct experiments. However, strain SPH-1 grown with protocatechuate, phenylacetate or succinate did

not show 4-sulfocatechol-dependent oxygen uptake, so we presume that the putative 4-sulfocatechol 1,2-dioxygenase is inducible in this organism.

3-C4-SPC-grown cells of *C. testosteroni* KF-1 (or of strain SPB-2) showed substrate-dependent oxygen uptake after addition of 3-C4-SPC, *p*-phenolsulfonate, 4-sulfocatechol or protocatechuate (Table 2), but not after addition of 2-C4-SPC, 4-C6-SPC, 4-sulfobenzoate or benzoate.

Substrate disappearance during the oxygen uptake was confirmed by HPLC (gradient system IV), and all reactions remained colourless. No substrate disappeared in the absence of oxygen (see below), but the addition of oxygen led to a reaction. We inferred that oxygenases were involved in these reactions. The oxygenases were absent in succinate-grown cells, so we presume that the enzymes are inducible.

TABLE 2. Specific activities of enzymes in whole cells of *C. testosteroni* KF-1 grown with different substrates.

Substrate in reaction mixture	Specific oxygen-uptake rates in reactions with cells grown with the named substrate (mkat/kg protein)			
	3-C4-SPC	4-Sulfophenol	Protocatechuate	Succinate
3-C4-SPC	1.1 [a]	1.2 [a,b]	0.1	0.4
2-C4-SPC	< 0.01	< 0.01	*n.d.*	*n.d.*
4-C6-SPC	< 0.01	< 0.01	*n.d.*	*n.d.*
4-Sulfophenol	0.1 [a]	0.1 [a,b]	< 0.01	< 0.01
4-Sulfocatechol	0.3 [a,c]	0.3 [a,b,c]	< 0.01	< 0.01
Protocatechuate	*n.d.*	0.4 [c]	2.4 [c]	-

[a] Substrate disappearance was confirmed by HPLC.

[b] Anoxic cell suspensions (N_2) did not convert the substrate, but the addition of air enabled a reaction.

[c] The reaction mixture developed no yellow colour.

n.d. Not determined.

3-C4-SPC-oxygenase, *p*-phenolsulfonate-oxygenase, 4-sulfocatechol-oxygenase and protocatechuate-oxygenase were detected when *p*-phenolsulfonate-grown cells of *C. testosteroni* KF-1 (or strain SPB-2) were examined (Table 2), whereas protocatechuate-grown cells exhibited a high level of protocatechuate-oxygenase only, again with a colourless reaction mixture. Growth with the commercially available *p*-phenolsulfonate allowed a preliminary examination of these oxygenases. Cell suspensions incubated under anoxic conditions (N_2-gas phase) did not degrade 3-C4-SPC, *p*-phenolsulfonate or 4-sulfocatechol until air was added to the reactions (not shown), so each reaction presumably involved an oxygenase.

The *ortho*-ring cleavage of 4-sulfocatechol was confirmed in crude extracts prepared from *p*-phenolsulfonate-grown cells of strain KF-1. Substrate disappearance was detected as a decrease in absorption at 238 nm and 283 nm, while the concomitant formation of 3-sulfo *cis*,*cis*-muconate was detected as an increase of absorption at 257 nm. The activity of 4-sulfocatechol 1,2-dioxygenase was determined to be about 0.2 mkat/kg protein in the oxygen electrode. 3-C4-SPC-oxygenase and *p*-phenolsulfonate oxygenase activities were not detected in crude extracts.

DISCUSSION

The data show that commercial LAS is converted to about 9 major SPCs (and many minor products) by *P. lavamentivorans*^{T} DS-1 (Fig. 2AB) and that each individual LAS congener, which we could test, was converted to one major SPC (and many minor products) (Fig. 3, Table 1). Where LAS congeners with even chain-lengths and the same substitution (position 2 on the chain) were available, the same major SPC was formed (Fig. 2AB), so we presume that this is true for each substituent position (2 to 7 in Fig. 1), and also for the odd chain-lengths for all 20 LAS congeners. The data (Table 1; see also Eichhorn and Knepper 2002; Dong *et al.* 2003) show that β-oxidation is the major pathway of chain-shortening, as observed

repeatedly (Swisher 1987; Schöberl 1989) by authors who formulated the 'distance principle', whereby the methyl group more distant from the substituent is attacked. Further, where the identity of the SPC has been thoroughly identified, as with 3-C4-SPC from 2-C10-LAS, from 2-C12-LAS and from 2-C14-LAS (Fig. 3, Table 1), or 4-C6-SPC from 3-C12-LAS (Schleheck *et al.* 2000), β-oxidation stops two or three carbon atoms distant from the substituent (when a even- or odd alkyl-side chain of LAS is degraded, respectively), and there is no evidence of movement of the substituent on the chain. This allows the major SPC generated from each LAS congener by strain DS-1^T to be predicted (Fig. 1). There are, thus, 11 major SPC products, one C4-SPC, and two each for the C5-, C6-, C7-, C8-, and C9-SPCs. The observation, about 9 major products from C4-SPC to C9-SPC (Fig. 2B), was estimated conservatively, given an uneven distribution of the positions of the substituent of commercial LAS (cf. Fig. 2A), and the generation of SPdCs from the long-chain SPCs (Dong *et al.* 2003), so the relative amounts of longer-chain SPCs may be lower than those of the smaller SPCs.

The metabolism sketched in Fig. 1 implies the presence of a large number of intermediates in *P. lavamentivorans*^{T} DS-1, almost none of which has been observed. The (mono)oxygenation of LAS is presumed, because no reaction occurs in the absence of oxygen (Swisher 1987; Schleheck, unpublished). The presumed product of oxygenation (the alcohol) has not been observed, and the aldehyde is presumed from standard biochemical reactions leading to the acid: this SPC has been observed, usually in complex matrices (González-Mazo *et al.* 1997; Hrsák and Begonja 2000; Dong *et al.* 2003). None of the many thioesters needed for β-oxidation has been observed, but the patterns of excreted intermediates (e.g. Table 1), especially the SPC-2H, make β-oxidation the only option (discussed in Dong *et al.* 2003). Much work remains to be done to confirm the biochemical reactions being catalysed by strain DS-1^T. In addition, there is no information on the transport mechanisms involved in the bringing LAS to the oxygenase, or the excretion of SPCs from the cytoplasm, where the β-oxidation enzymes may be expected.

The SPC-2Hs, which we observed (Table 1), are presumed to carry the Δ-2 double bond (Fig. 4, Fig. 5), which gives a wider delocalisation of π-electrons and the shift of the UV-spectrum to longer wavelengths in the example shown (Fig. 5). The fact that 3-C5-SPC, 5-C7-SPC and 4-C5-SPC also give this UV-spectrum, where the Δ-2 double bond should not lead to delocalisation of electrons into the ring, shows that these compounds need to be isolated and examined in more detail to establish their identity and their generation.

Whereas most of the LAS was metabolised by strain DS-1^T via the β-oxygenation pathway, a small portion (< 5 %) may have been subject to α-oxidation (Table 1). This reaction, however catalysed, is readily seen in some environmental compartments (León and González-Mazo 2003). α-Oxidation can presumably be studied best in an isolated organism which uses the reaction as the major pathway to degrade LAS.

The degradability of SPCs, SPdCs, and the recently discovered SPC-2Hs has been recognised implicitly for many years, because LAS is fully biodegradable (e.g. Kölbener *et al.* 1995a; Kölbener *et al.* 1995b), and degradative organisms were isolated in laboratories which did not have extensive analytical capabilities to explore their physiology in detail (Jiménez *et al.* 1991; Sigoillot and Nguyen 1992; Hrsák and Begonja 1998). Schulz et al. (Schulz *et al.* 2000) found that their isolate, *D. acidovorans* SPB1, has a very narrow substrate spectrum for SPCs, namely *RS*-2-C4-SPC, which are degraded sequentially, the *S*-enantiomer first. The new isolates, *D. acidovorans* SPH-1, and *C. testosteroni* strains SPB-2 and KF-1, also have narrow substrate ranges for SPCs, but they seem to degrade three to four compounds each: 4-C6-SPC, 4-C6-SPC-2H, 4-C5-SPC, and 4-C5-SPC-2H for strain SPH-1; 3-C4-SPC, 3-C4-SPC-2H, and 3-C5-SPC for strains SPB-2 and KF-1. *D- acidovorans* SPH-1 utilises *RS*-4-C6-SPC enantiomer-specifically, in that the putative *S*-enantiomer is utilised first (Fig. 7), and preliminary data from H.-P. E. Kohler (EAWAG, Dübendorf, Switzerland; personal communication) indicate that *C. testosteroni* KF-1 utilises 3-C4-SPC enantiomer-specifically. It seems likely that many SPCs are utilised enantiomer-specifically, but the mechanisms involved (two enantiomer-specific transport

systems and two enantiomer-specific oxygenases in ref. Kohler 1999) remain to be explored. Transport systems for organosulfonates are axomatic (Graham *et al.* 2002), and the first transport system for an arenesulfonate is being elucidated in *C. testosteroni* T-2 (Tralau *et al.* 2003a; Mampel et al., in preparation). The first reaction in the catabolism of e.g. 3-C4-SPC in strain KF-1 is an inducible oxygenase(s) (Table 2), which is inactive in our cell-free preparations.

The degradative pathway(s) for SPCs was the subject of much speculation (e.g. Cook 1998) till experimental data became available. Schulz et al. (Schulz *et al.* 2000) showed the involvement of inducible 4-sulfocatechol 1,2-dioxygenase in the degradation of 3-C4-SPC and Dong et al. (Dong *et al.* 2003) found high activity of the enzyme in a mixed culture which degraded SP(d)Cs. The new isolates, strains SPH-1, SPB-2 and KF-1, all express 4-sulfocatechol-1,2-dioxygenase inducibly during the degradation of the appropriate SPC (Table 2 and Results), so we consider it highly likely that all SPCs are degraded via 4-sulfocatechol and the *ortho* pathway elucidated by Feigel and Knackmuss (Feigel and Knackmuss 1993; Contzen *et al.* 2001), whose central reactions, including desulfonation, are shown in Fig. 5. Further work on this *ortho* pathway showed that the ring cleavage enzyme of *Hydrogenophaga intermedia* S1 is almost identical with the 3,4-dihydroxybenzoate 3,4-dioxygenase in the same organism: whereas the 3,4-dihydroxybenzoate dioxygenase cleaves only 3,4-dihydroxybenzoate, the 4-sulfocatechol-1,2-dioxygenase can cleave both 3,4-dihydroxybenzoate and 4-sulfocatechol (Contzen *et al.* 2001). The same phenomenon is observed in *C. testosteroni* KF-1 (Table 2) and in strain SPB-2.

The shortages of SPCs and of 4-sulfocatechol led us to explore 4-sulfophenol as a potential, commercially available, growth substrate with which to obtain 4-sulfocatechol-1,2-dioxygenase. Whereas strain SPH-1 failed to grow, strains SPB-2 and KF-1 grew with 4-sulfophenol and expressed 4-sulfocatechol-1,2-dioxygenase, as hoped (Table 2). Presumably 4-sulfophenol is oxygenated by a 4-sulfophenol-2-monooxygenase (Table 2) to 4-sulfocatechol.

This is the first demonstration of a degradative pathway for 4-sulfophenol, whose degradation was first observed in the 1960s (Symons and del Valle-Rivera 1961).

Growth of *C. testosteroni* KF-1 (or strain SPB-2) with 4-sulfophenol gratuitously induces the 3-C4-SPC oxygenase(s) (Table 2). The simplest degradative pathway for 3-C4-SPC that we can postulate is an oxygenolytic cleavage of the sidechain to yield 4-sulfophenol and a C4-carboxylic acid. The latter is utilised for growth, as indicated by the molar growth yield for 3-C4-SPC of about 6 g protein/mol C. The former is oxidised via 4-sulfophenol-2-monooxygenase. We know of no precedent for the reaction postulated for 3-C4-SPC, except the cleavage of 2-C4-SPC in strain SPB1 (Schulz *et al.* 2000) and the corresponding reactions with 3-C4-SPC in strain SPB-2 and with 4-C6-SPC in strain SPH-1 (this paper).

C. testosteroni KF-1 grew uniformly in suspended culture with 3-C4-SPC, 4-sulfophenol and compounds like benzoate, but when small amounts of LAS, which the organism does not attack, were added to the culture, the organism started to form a biofilm. Presumably this is a stress response, which is also detected in strains SPB-2 and SPH-1. *P. lavamentivorans*^{T} DS-1 degrades LAS only when it can form a biofilm, so the biofilm in LAS-degrading co-cultures of e.g. strains KF-1 and DS-1^{T} is presumably contributed by both organisms.

The representative degraders of SPCs, strains SPH-1 and KF-1 (or strain SPB-2), degrade four (Fig. 2E) of the 11 major SPCs (Fig. 1) produced from commercial LAS by strain DS-1^{T}. These SPCs thus represent the main products from 8 of the 20 LAS congeners. If this specificity of SPC-degraders is generally true, at least four more organisms are needed to degrade the other major SPCs that are detected. We have no evidence for the degradation of SPdCs under these conditions (Fig. 2), so many more organisms are presumably needed to degrade the minor components of the mixture of compounds generated from LAS by strain DS-1^{T}. The ready biodegradability of commercial LAS masks a complex community of microorganisms and many poorly understood biochemical reactions.

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CHAPTER 6

Parvibaculum lavamentivorans^{T} DS-1 degrades centrally-substituted congeners of commercial linear alkylbenzenesulfonate (LAS) into sulfophenylcarboxylates and sulfophenyldicarboxylates

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ABSTRACT

Parvibaculum lavamentivorans^{T} DS-1 degrades single, laterally-substituted congeners of linear alkylbenzenesulfonate (LAS) (e.g. 2-(4-sulfophenyl)decane, 2-C10-LAS) through ω-oxygenation and β-oxidation to a major sulfophenylcarboxylate (SPC, 3-C4-SPC, about 80 % of total products) and two minor products, an α,β-unsaturated SPC (SPC-2H, 3-C4-SPC-2H), and the SPC species from the earlier round of β-oxidation (SPC+2C; 5-C6-SPC)); no sulfophenyl-dicarboxylate (SPdC) is detected. Degradation of all 20 congeners of commercial LAS is predicted to generate 11 major SPCs, with minor products which include SPdCs from the centrally-substituted LAS congeners (D. Schleheck, T. P. Knepper, K. Fischer and A. M. Cook, Appl. Environ. Microbiol. submitted). The degradation of 5-C10-LAS yielded largely 4-C8-SPC, with 4-C8-SPC-2H and 6-C10-SPC (about 70 % of products), together with C6-, C8-, and C10-SPdCs, as identified by mass spectrometry coupled to high-pressure liquid chromatography (HPLC). The degradation of 6- and 5-C12-LAS, which were separated from commercial LAS by semi-preparative HPLC, yielded mainly C8-SPCs, with SPC-2Hs and SPC+2Cs (about 60 % of products), and C6-, C8-, C10- and C12-SPdCs. The preparation of 7-, 6- and 5-C13-LAS was degraded mainly to C9- and C7-SPC species (and minor components; about 50 % of products), and to C5-, C7-, C9- and C11-SPdCs. Solely SPCs, but no detectable SPdC, were generated from 4-C12-LAS and from 4-C13-LAS. The range of products generated from commercial LAS is considered to comprise the 11 major SPCs, each with a minor SPC-2H and a SPC+2C, i.e. 33 individual SPC and SPC-2H species, with up to 17 different SPdC species. Thus, a large array of compounds, many in low quantities, is generated by strain DS-1^{T} during degradation of commercial LAS.

INTRODUCTION

The major laundry surfactant in world wide use is commercial linear alkyl-benzenesulfonate (LAS) (e.g. Schulze 1996). The preparation is nominally a mixture of 20 congeners (Kosswig 1994), which are the C_{10} - C_{13}-*n*-alkanes with a subterminal 4-sulfophenyl substituent, and 18 congeners are present in racemic mixture. Commercial LAS thus represents 38 individual compounds (cf. Kölbener *et al.* 1996; di Corcia *et al.* 1999b).

LAS is fully biodegradable in oxic environments, as first demonstrated in 1957 (Sawyer and Ryckman 1957, cf. Schöberl 1989). Degradation involves microbial communities, which can now be examined in defined mixed cultures (Hrsák and Begonja 1998; Schleheck *et al.* 2003c), whose transient extracellular intermediates include sulfophenylcarboxylates (SPCs) (e.g. Schöberl 1989), sulfophenyldicarboxylates (SPdCs) (e.g. di Corcia *et al.* 1999a; Dong *et al.* 2003) and α,β-unsaturated SPCs (SPC-2H) (Eichhorn and Knepper 2002; Dong *et al.* 2003; Schleheck *et al.* 2003c).

Parvibaculum lavamentivorans^T DS-1 (Schleheck *et al.* 2000; Schleheck *et al.* 2003e) is apparently representative of a member of many heterotrophic LAS-degrading communities (Dong *et al.* 2003) and it catalyses the first steps of LAS-degradation (Schleheck *et al.* 2003c). LAS is activated by ω-oxygenation of at least one terminal methyl group, a reaction catalysed by a P450 monooxygenase (Schleheck and Cook 2003b), and oxidised to the corresponding SPC (Dong *et al.* 2003), which is subject to β-oxidation (Dong *et al.* 2003; Schleheck *et al.* 2003c). β-Oxidation ceases at a distance of 3 or 4 carbon atoms from the 4-sulfophenyl substituent ((Schleheck *et al.* 2003c). A wide range of SPCs (C_4–C_9) (the major product in each case tested), SPC-2Hs, and SPdCs is excreted (Dong *et al.* 2003; Schleheck *et al.* 2003c). The mixture of those products from commercial LAS is termed SP(d)C.

Strain DS-1^T generates several SPC species from any one LAS congener (Dong *et al.* 2003; Schleheck *et al.* 2003c), a fact which was first recognised when single LAS congeners (e.g. 2-C10-LAS in Fig. 1) were supplied to strain DS-1^T, and products could be identified by high pressure liquid chromatography coupled by an electrospray interface to a mass spectrometer

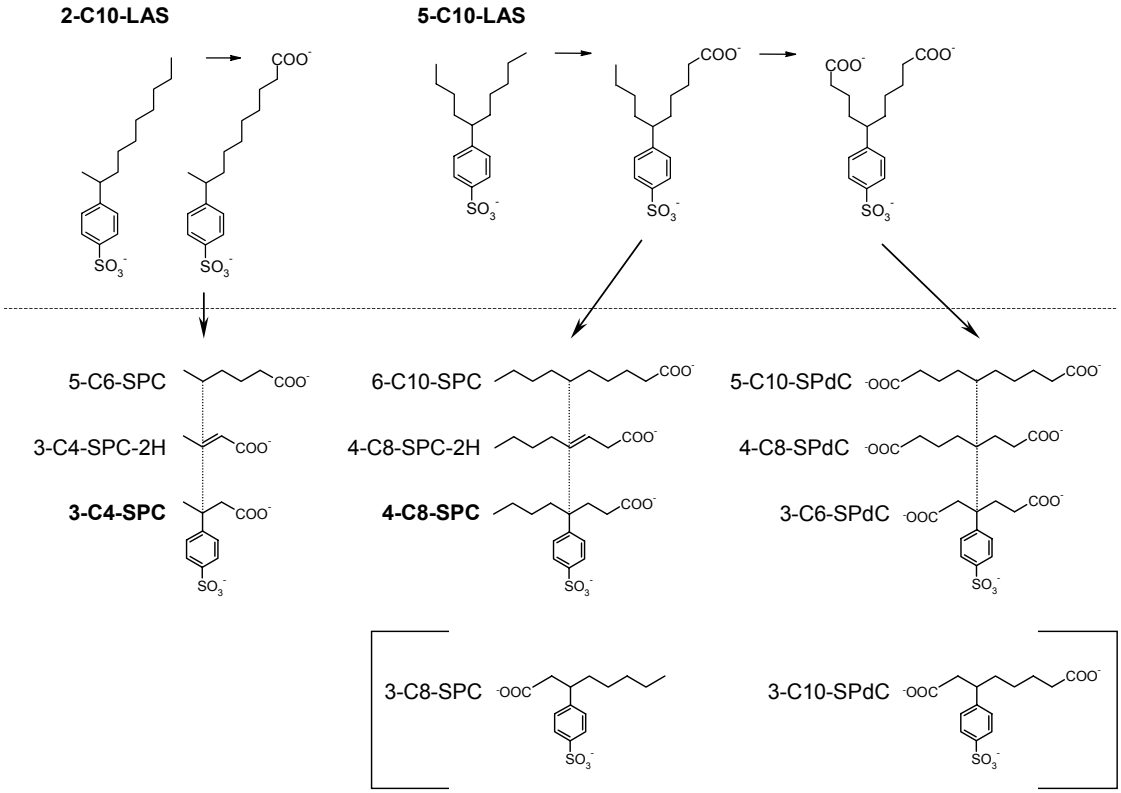


FIG. 1. SPCs and SPC-2H generated during growth of *P. lavamentivorans*^{T} DS-1 with a laterally-substituted LAS congener (2-C10-LAS), and SPCs, SPC-2H, and SPdCs generated during growth with a centrally-substituted LAS congener (5-C10-LAS). The LAS congeners are transformed to SPCs and SPdCs *via* ω-oxygenation(s) and ω-oxidation. The intermediates, presumably as CoA thioesters, are subject to β-oxidation and presumably de-esterification. SPCs, SPC-2Hs and SPdCs are excreted into the culture fluid during growth. The set of SPCs generated from 2-C10-LAS was identified in previous work (Schleheck *et al.* 2003c), and the set of SPC and SPdC from 5-C10-LAS in this work (see text). The SPC, which is excreted in major amounts (major SPC) is indicated in **bold** letters. Products, which are feasible, but which are apparently not generated in significant amounts, are shown in the [square brackets]. Molecular structures of SPC-2H species are drawn as inferred from the UV-scans of the parent compounds (see text).

(HPLC-ES-MS). One major SPC species is formed (e.g. 3-(4-sulfophenyl)butyrate, 3-C4-SPC, in Fig. 1), with the corresponding SPC-2H (3-C4-SPC-2H in Fig. 1), and the SPC from an earlier round of β-oxidation (SPC+2C) (5-C6-SPC in Fig. 1). The data with laterally-substituted LAS congeners allowed the prediction that 11 major SPC are generated from the 20 LAS congeners, one C4-SPC, and two C5-, C6-, C7-, C8-, and C9-SPCs, and several of these compounds were observed (Schleheck *et al.* 2003c). The generation of SPdCs (Dong *et al.* 2003) was thus assumed to result from the degradation of centrally-substituted LAS congeners.

We now confirm the prediction that 11 major SPCs are generated from commercial LAS, by observing the previously unknown compounds. The corresponding SPC-2Hs and SPC+2Cs were also detected. SPdCs are shown to be generated from the degradation of centrally-substituted LAS congeners. These data allow us to predict all SPC, SPC-2H, SPC+2C and SPdC-species generated from commercial LAS by strain DS-1^T.

MATERIAL AND METHODS

Chemicals

The commercial LAS in use was Marlon A350 (Schleheck *et al.* 2000). 5-C10-LAS (> 93 %) was a gift from César Bengoechea (Petresa, Madrid, Spain). The sources of 2-C10-LAS, 2-C11-LAS, 2-C12-LAS, 3-C12-LAS, and, if appropriate, the nature of impurities, are described elsewhere (Dong *et al.* 2003; Schleheck *et al.* 2003c).

Analytical methods

Gradient system I (0.11 M perchlorate/acetonitrile, Schleheck *et al.* 2000) was used for HPLC coupled to a diode array detector (HPLC-UV). The retention times of representative C4-C8-SPCs were known from previous work (Schleheck *et al.* 2003c). Gradient system II was used for HPLC separations involving the electrospray interface coupled to the mass spectrometer as detector (ES-MS) operating in the negative ion mode (HPLC-ES-MS); the mobile phase included the volatile ion pair reagent acetic acid/triethanolamine (5 mM) (Eichhorn and Knepper 2002). SPC, SPC-2H, and SPdC species were tentatively identified by the *m/z*-value of the deprotonated molecular ion, and the identity was confirmed by specific fragmentation patterns, as described by Eichhorn and Knepper (Eichhorn and Knepper 2002).

The HPLC-ES-MS chromatograms were from analyses with programs that scanned for deprotonated molecular ions of SPCs (C4-C13, *m/z*-values 243, 257, 271, 285, 299, 313, 327, 341, 355, 369) and SPC-2Hs (C4-C13, *m/z*-values 241, 255, 269, 283, 297, 311, 325, 339, 353, 367) in a first run, and for SPdCs (C4-C13, *m/z*-values 273, 287, 301, 315, 329, 343, 357, 371, 385, 399) in a second run; the separate chromatograms were used to reduce the complexity of the data sets. For the same reason, we did not scan for molecular ions corresponding to putative SPdC-2Hs (C4-C13, *m/z*/values 271, 285, 299, 313, 327, 341, 355, 369) whose *m/z*-values are identical with those of many SPCs. No authentic standard for SPdC was available.

Semi-preparative HPLC

Semi-preparative HPLC was done as described previously (Schleheck *et al.* 2003c), with gradient system I. LAS eluted in four sets of peaks corresponding to the isomers of the C10-, C11-, C12-, and C13-alkyl chains: centrally-substituted isomers eluted before laterally-substituted compounds (e.g. Kölbener *et al.* 1995a), but there was no baseline-separation, except for the 2-substituted congener. The first stage of separation separated the groups of isomers from one another, whereby the 2-substituted congener was also separated. Each set of isomers was rechromatographed, partly to remove homologues and partly to cut the isomers into four fractions, each with a different major LAS congener (see e.g. Fig. 2BCD). Each fraction was subject to treatment in a Rotavap, to remove the acetonitrile, then desalted and concentrated by solid-phase extraction (Schleheck *et al.* 2003c).

Degradation of LAS by *P. lavamentivorans*^{T} DS-1

Salts-medium with a solid support (1 mg/ml glass particles) for biofilm formation was supplemented with LAS to 0.2 mM (determined as total $A_{220\ nm}$) and inoculated with *P. lavamentivorans* (DSM 13023^{T}, NCIMB 13966^{T}) (Schleheck *et al.* 2003e). Degradation of LAS during growth was assayed as the disappearance of foam (Schleheck *et al.* 2003e). Samples were taken from the culture medium before and after growth and analysed by HPLC-UV and by HPLC-ES-MS.

RESULTS AND DISCUSSION

SPC and SPdC generated during degradation of 5-C10-LAS

P. lavamentivorans^{T} DS-1 grew reproducibly with 5-C10-LAS in salts medium supplemented with glass particles (Schleheck *et al.* 2003e) if the concentration was ≤ 0.2 mM. There was no growth with 1 mM 5-C10-LAS, whereas laterally-substituted congeners at this concentration supported rapid growth (Schleheck *et al.* 2003e). We infer that 5-C10-LAS (with other centrally substituted LAS-congeners) is more toxic than the laterally-substituted congeners, so the routine total concentration of LAS in growth media in this work was 0.2 mM.

HPLC-UV analyses of samples taken before and after growth of strain DS-1^{T} with 5-C10-LAS (Fig. 2A) showed the formation of three major products (peaks at 11.8, 12.6, and 14.3 min), which are SPCs and a SPC-2H (see below), and several minor products (peaks at retention time < 11.0 min), which largely represented SPdCs (see below). All products had SPC-typical UV-scans (not shown), with the exception of the peak at 11.8 min (see below). An elution profile with three major products was obtained by HPLC-ES-MS, when scanned for molecular ions corresponding to SPCs and SPC-2Hs (Fig. 3A), and the mass signals obtained could be assigned to the major peaks observed by HPLC-UV (Fig. 2A): the major peak at 12.6 min retention time represented a C8-SPC species; the second major peak at 14.3 min was a C10-SPC; the peak at 11.8 min showed a mass signal corresponding to C8-SPC-2H. The traces of other SPC species detected (Fig. 3A) were attributed to degradation of the impurity (2-C10-LAS) in the preparation (2-C4-SPC and 4-C6-SPC, at 8.0 and 10.4 min in Fig. 2AD), whereas other impurities were apparently not degraded (Fig. 2A). A C9-SPC (Fig. 3) was detected in low amounts (trace peak at 13.2 min in Fig. 2A; see below).

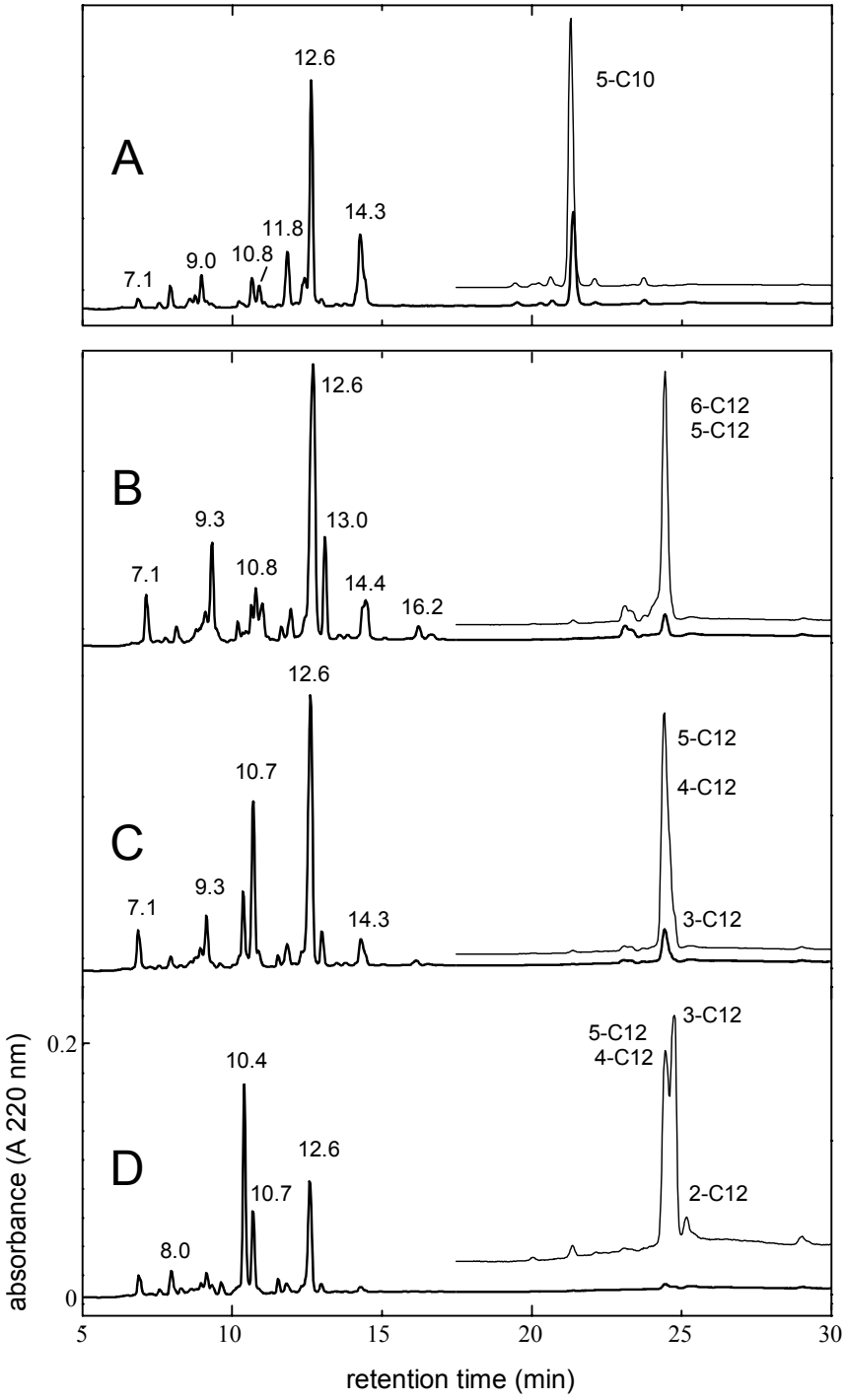


FIG. 2. HPLC-UV chromatograms of LAS in salts-medium, and of products detected after growth of strain DS-1^T with different congeners of LAS. The separation was done with gradient system I, and no relevant peaks were observed at retention time < 5 min. The upper curve in any panel represents the LAS preparation used in that experiment. For simplicity, only the LAS-region is shown, and the congeners present are indicated; their identity was confirmed by HPLC-ES-MS. The retention times (min) of the largest product-peaks are shown.

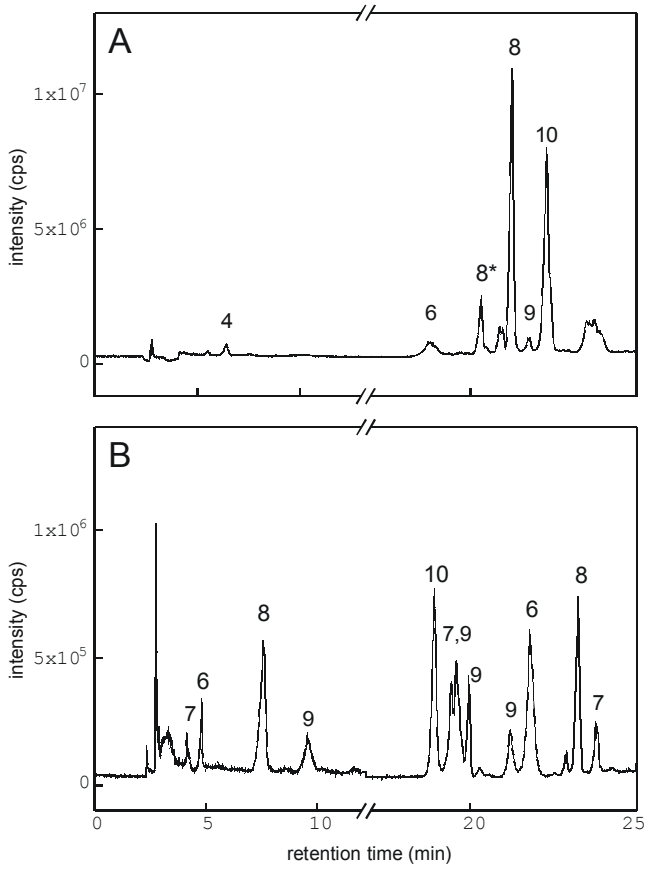


FIG. 3. HPLC-ES-MS chromatograms of compounds from culture medium sampled after growth of strain DS-1^T with 5-C10-LAS (cf. Fig. 2A). The chromatograms resulted from scanning for deprotonated molecular ions of SPC and SPC-2H (A) and SPdC (B). The numbers give the chain-length of the compound, derived from the mass spectrum. *, Mass signal corresponding to SPC-2H species.

We concluded that the products from this centrally-substituted LAS congener, 5-C10-LAS, largely represented the pattern observed for the laterally-substituted LAS congeners, with a major and a minor SPC and a SPC-2H (see Introduction), which together represented about 73 % of the total products (6.8–14.5 min). The major SPC was presumed to be 4-C8-SPC (cf. Schleheck *et al.* 2003c) (12.6 min in Fig. 2A), the minor SPC (SPC+2C) must be 6-C10-SPC (14.3 min in Fig. 2A) and the second minor product (SPC-2H) is 4-C8-SPC-2H (11.8 min in Fig. 2A).

This 6-C10-SPC reconfirmed ω-oxygenation of the LAS. β-Oxidation was reconfirmed as the major mechanism of chain-shortening with 4-C8-SPC, and especially 4-C8-SPC-2H, as the other major products (discussed in Dong *et al.* 2003), and the argument is strengthened by the SPdCs which are also characterised (below).

The generation of 3-C8-SPC (peak at 13.0 min in Fig. 2 AB, see also below) from 5-C10-LAS was detectable, but it was not a major product. This indicated that the (first) ω-oxygenation proceeded almost exclusively at the longer alkyl-side chain of 5-C10-LAS, at the C5-sidechain, yielding 4-C8-SPC as major SPC, and not 3-C8-SPC (Fig. 1). We presume this observation, which is a version of `Swisher's distance principle´ (Swisher 1987), to be generally valid (see below).

The data depicting separations of SPdCs (Fig. 3B) show peaks with intensities about 10 % of those obtained for SPCs (Fig. 3A): this is in rough agreement with the smaller peaks (7-11 min) in Fig. 2A. The distribution of the tentatively identified signals (Fig. 3B) would appear anomalous, firstly because there are many more signals of similar size than indicated in Fig. 2A, and secondly, the signals for C6- to C10-SPdC in the range 5-18 min would appear to be recur from 19- >25 min. Indeed, there would appear to be four different C9-SPdCs (Fig. 3B), where α-oxidation is rare, and there is no possibility to generate more than two such species (see Fig. 1). We presume that gradient system II is not robust when separating the tri-basic SPdCs in HPLC-ES-MS, possibly as a function of the ion-pair reagent, but we feel prepared to interpret the data in the peaks eluting prior to 18 min.

The presence of C10-SPdC confirmed that the ω-oxygenation of the second, terminal methyl group of 5-C10-LAS is feasible, i.e. at the C4-side chain of 6-C10-SPC (Fig. 1), and that this ω-oxygenation can proceed before any β-oxidation occurs. β-Oxidation apparently led to the formation of C8-SPdC, and to the trace of C6-SPdC (Fig. 3B). Given that β-oxidation stops at least three carbon atoms distant from the sulfophenyl substituent (Schleheck *et al.* 2003c and above), each of the two carboxylates generated by ω-oxygenation was subject to β-oxidation.

Both C9-SPdC and C7-SPdC were detected. These presumably arose by the often-mentioned but as yet undefined α-oxidation of LAS (or SPC?), which is seen and discussed elsewhere (Schleheck *et al.* 2003c).

The UV-spectrum of putative C8-SPC-2H (maximum at 264 nm) was identical with those of other SPC-2Hs (Schleheck *et al.* 2003c) and it differed from the UV-spectrum of SPCs (e.g. maximum at 221 nm for 4-C8-SPC). This shift to higher wavelengths, and the implied wider delocalization of π-electrons than in SPC, leads us to draw the structure with a Δ-3 double bond (Fig. 1), rather than with the Δ-2 double bond expected of β-oxidation (see adjacent 3-C4-SPC-2H in Fig. 1). More of this compound must be generated to establish its structure, and whether an isomerase is present to catalyse its formation (Schleheck *et al.* 2003c).

SPC and SPdC generated from 6-C12-LAS and 5-C12-LAS

The mixture of 6- and 5-C12-LAS was degraded by strain DS-1^T, and the products were analysed by HPLC-UV (Fig. 2B) and HPLC-ES-MS (Fig. 4). The three largest peaks obtained from 5-C10-LAS, 4-C8-SPC (12.6 min in Fig. 2B), 6-C10-SPC (14.4 min in Fig. 2B) and 4-C8-SPC-2H (11.8 in Fig. 2B) were attributed to degradation of 5-C12-LAS, analogous to earlier work where the products from 2-C10-LAS, from 2-C12-LAS, and from 2-C14-LAS were identical (Schleheck *et al.* 2003c). The dominant peak at retention time 13.0 min (Fig. 2B) could thus be attributed to metabolism of 6-C12-LAS, and the peak presumably represented 3-C8-SPC as the major SPC from 6-C12-LAS. Analysis by HPLC-ES-MS indicated C12-SPC(s), C10-SPC(s) and C8-SPC(s) (Fig. 4A). The less efficient separation of SPCs in gradient system II, compared with system I (Schleheck *et al.* 2003c) presumably explains the single peak of C8-SPC(s), where two peaks are attributed to different C8-SPCs in Fig. 2B. The peak clusters with retention times at about 14.4 min and 16.2 min in Fig. 2B were deduced to represented C10- and C12-SPCs, respectively (Fig. 4A). The broad peak at 14.4 min presumably contains 6-C10-SPC (from 5-C12-LAS) and 5-C10-SPC (from 6-C12-LAS), in the class of SPC+2Cs. The peak(s) at

about 16.2 min were apparently SPCs generated by ω-oxygenation and oxidation to the carboxylate; the major components were presumed to be 8-C12-SPC (from 5-C12-LAS) and 7-C12-SPC (from 6-C12-LAS). The class of SPC-2Hs was also generated from 5- and 6-C12-LAS (Fig. 4A), though only 4-C8-SPC-2H was located in Fig. 2B (11.8 min).

The presence of C9-SPC and C7-SPC, with a C7-SPC-2H (Fig. 4) was attributed to the degradation of an impurity (a C11-LAS) in the preparation (Fig 2B). The contribution of these identified SPCs to the total amount of products from 6- and 5-C12-LAS was about 65 %. HPLC-ES-MS analyses showed the presence of C6-, C8-, C10- and C12-SPdCs, with a trace of C9-SPdC (Fig. 4); we attribute the peak at 22 min to the analytical problems discussed above.

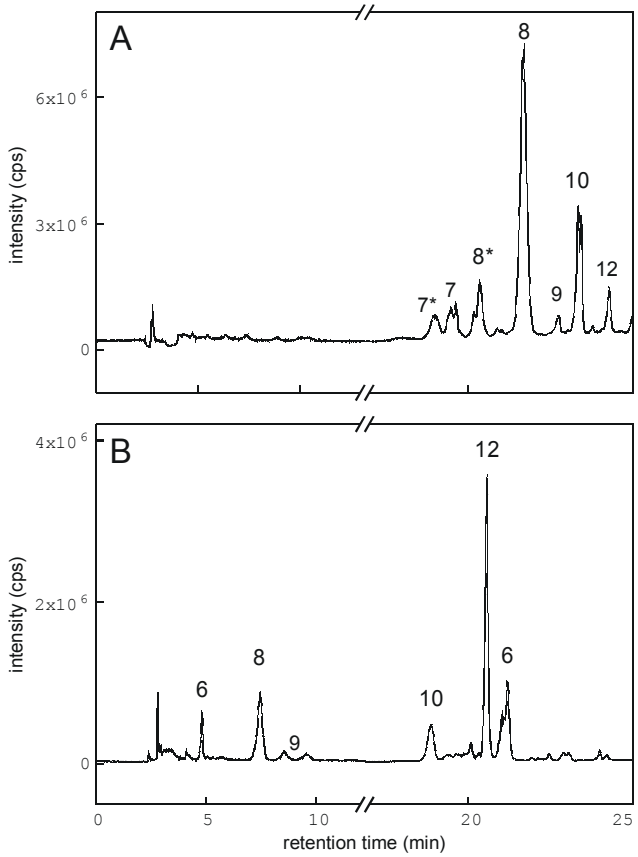


FIG. 4. HPLC-MS chromatograms of culture medium after growth of strain DS-1^T with 6- and 5-C12-LAS (cf. Fig. 2B). The chromatograms resulted from scanning for deprotonated molecular ions of SPC and SPC-2H (A) and SPdC (B). The numbers give the chain-length of the compound, derived from the mass spectrum. *, Mass signals corresponding to SPC-2H species.

C6-, C8- and C10-SPdCs (Fig. 4B) could be assigned to the peaks at retention time 7.1 min, 9.3 min and 10.8 min, respectively, in the HPLC-UV-chromatogram (Fig. 2B); the C12-SPdC presumably co-eluted with an SPC in gradient system I.

5-C12-LAS and 4-C12-LAS with low amounts of 3-C12-LAS and traces of 6-C12-LAS were utilised by strain DS-1^T (Fig. 2C), but the pattern of products was much simpler than the previous example (Fig. 2B). The 4-C8-SPC, 6-C10-SPC and 4-C8-SPC-2H (12.6, 14.3 and 11.8 min) from 5-C12-LAS were present, as were the C6-, C8- and C10-SPdCs (7.1, 9.3 and 10.8 min), though at lower abundance than in Fig. 2B. Putative 3-C8-SPC (13.0 min), whether from 6-C12-LAS or from α-oxidation of 5-C12-LAS, was much reduced compared to Fig. 2B. There was a new major peak, at 10.7 min (Fig. 2C) which represented a C6-SPC species (HPLC-ES-MS, not shown), presumably 3-C6-SPC from 4-C12-SPC. The peak at 10.4 min (Fig. 2C) was 4-C6-SPC from 3-C12-LAS, and it was identified by co-chromatography with authentic material (cf. Schleheck 2000). Given the simplicity of the chromatogram (Fig. 2C), there are no significant unidentified peaks, so, apparently, no SPdC was generated from 4-C12-LAS. This is interpreted to mean that ω-oxygenation does not occur at C3-side chains of LAS (or C3-sidechains of SPC).

The mixture of 5-C12-LAS, 4-C12-LAS, 3-C12-LAS, and 2-C12-LAS (Fig. 2D) was utilised by strain DS-1^T. The organism excreted 4-C8-SPC (12.8 min from 5-C12-LAS; Fig. 2D), 3-C6-SPC (10.7 min from 4-C12-LAS), 4-C6-SPC (10.4 min from 3-C12-LAS), and 3-C4-SPC (8.0 min from 2-C12-LAS), which was identified by co-chromatography (and HPLC-ES-MS) with material generated elsewhere (Schleheck *et al*. 2003c). The corresponding peaks for SPC+2C and SPC-2H were all detected, as were the SPdCs from 5-C12-LAS. There is, indeed, a reproducible pattern in the degradation of the LAS congeners. Another generalisation is that when positional isomers, whether of LAS or of SPC, are separated by HPLC, the longer the uninterrupted alkyl chain, the longer the retention time (e.g. Fig. 2D for LAS and for the C6-SPCs).

SPC and SPdC generated from 7-C13-LAS, 6-C13-LAS and 5-C13-LAS

7-C13-LAS, 6-C13-LAS, and 5-C13-LAS, which were not separated by HPLC (Fig. 5A), were utilised by strain DS-1^T (Fig. 5A). The most abundant material indicated by HPLC-ES-MS was C9-SPC(s) (Fig. 6A), which was poorly separated into at least two peaks by gradient system I (peaks at 13.5–13.6 min in Fig. 5AB). The formation of two C9-SPC was predicted (Schleheck *et al.* 2003c), 3-C9-SPC (presumably the peak at 13.6 min) as the major SPC from 7-C13-LAS, and 4-C9-SPC (presumably the peak at 13.5 min) as the major SPC from 6-C13-LAS. Low levels of SPC+2C (15.3 min in Fig. 5A) and SPC-2H were detected by HPLC-ES-MS, as was C13-SPC from the ω-oxygenation and oxidation (Fig. 6A). The dominant C7-SPC species (Fig. 6) was attributed to the major SPC generated from 5-C13-LAS, putative 3-C7-SPC (11.9 min in Fig. 5A). The signals for C8- and C10-SPC (Fig. 6A) possibly derived from a minor impurity of C12-LAS. The tentatively identified SPCs represented about 52 % of the products generated.

HPLC-ES-MS analyses (Fig. 6B; peaks between 5 and 22 min were used; see above) indicated traces of C5-and C7-SPdCs and significant levels of C9-, C11- and especially C13-SPdCs (Fig. 6B). The signals corresponding to C6-, C8- and C10-SPdC were attributed to the impurities in the LAS-preparation. We attributed the peak at 13.2 min (Fig. 5A) to C13-SPdC, the peak at about 11.8 min to the C11-SPdCs, the peaks at 10.0 and 10.2 min to the C9-SPdCs, the peak at 7.9 min to the C7-SPdC, and the trace peak at 5.1 min to C5-SPdC(s).

The tentative identifications were supported by analyses of the products from the material containing some 7-C13-LAS and 6-C13-LAS in 5-C13-LAS with 4-C13-LAS and a trace of C3-C13-LAS (Fig. 5B). The lower amounts of 7-C13-LAS and 6-C13-LAS corresponded to the lower amounts of putative 3-C9-SPC and 4-C9-SPC (13.5 and 13.6 min, Fig. 5B) compared with Fig. 5A. The higher concentration of 5-C13-LAS and the presence of 4-C13-LAS were reflected in a higher concentration of putative 3-C7-SPC (11.9 min in Fig. 5B) and the appearance of

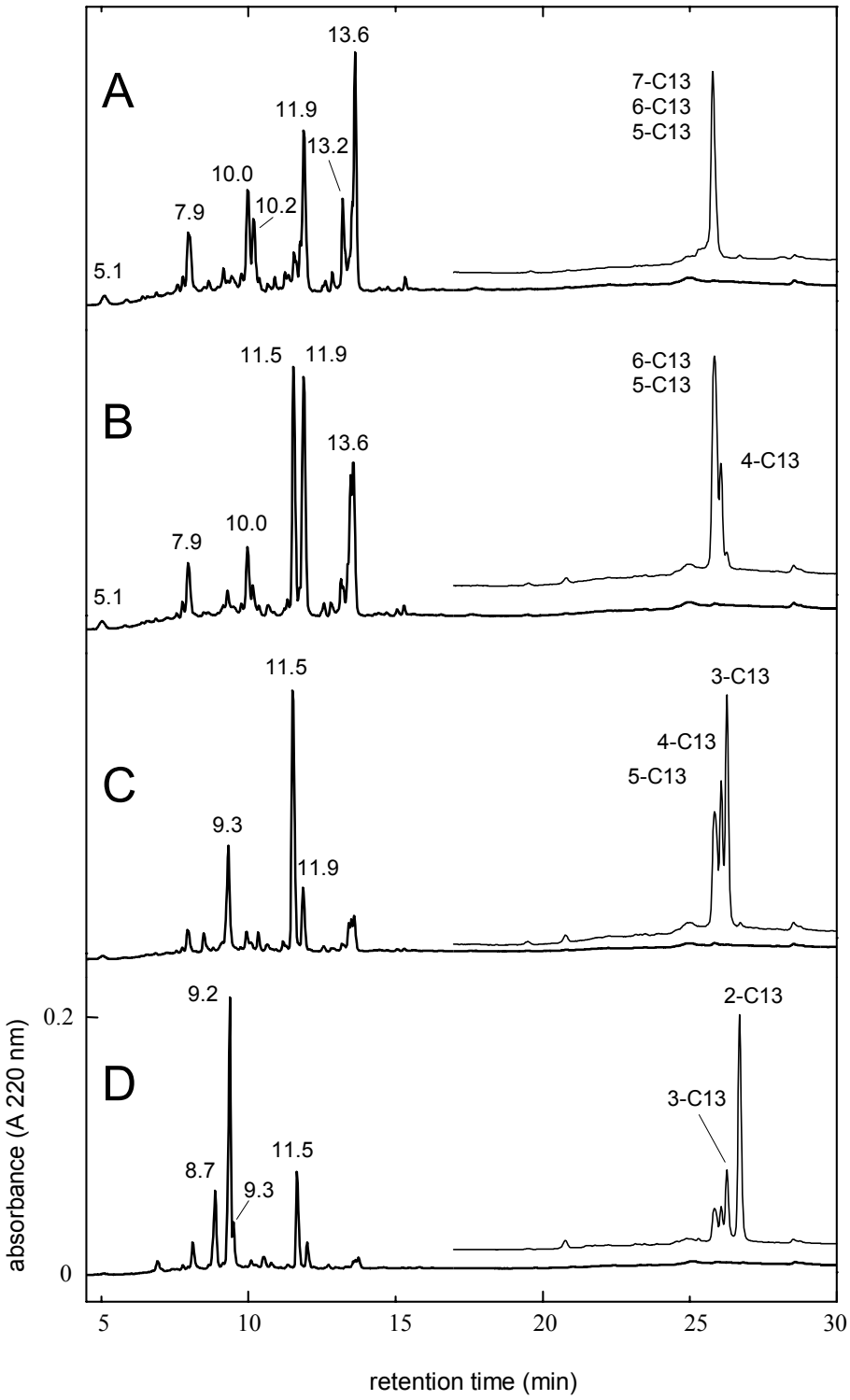


FIG. 5. HPLC-UV chromatograms of C13-LAS material as derived from preparative HPLC in culture medium, and of SP(d)C generated after growth of strain DS-1^T. The separation was done with gradient system I, and no relevant peaks were observed at retention time < 4.5 min. The upper curve in any panel represents the LAS preparation used in that experiment. For simplicity, only the LAS-region is shown, and the congeners present are indicated; their identity was confirmed by HPLC-ES-MS. The retention times (min) of the largest product-peaks are shown.

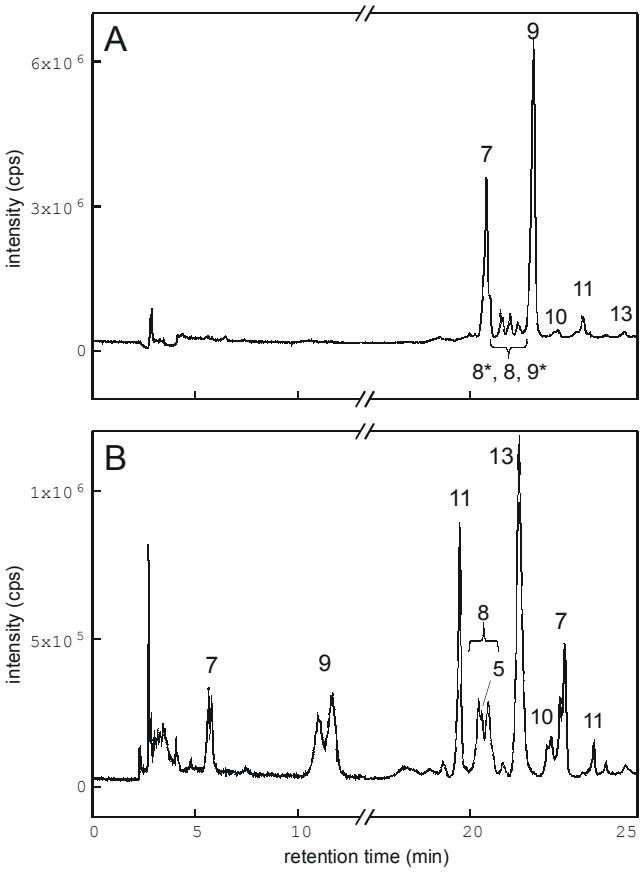


FIG. 6. HPLC-ES-MS chromatograms of culture medium after growth of strain DS-1^T with 7-, 6-, and 5-C13-LAS (cf. Fig. 5A). The chromatograms resulted from scanning for deprotonated molecular ions of SPC and SPC-2H (A) and SPdC (B). The numbers give the chain-length of the compound, derived from the mass spectrum. *, Mass signals corresponding to SPC-2H species.

putative 4-C7-SPC (11.5 min in Fig. 5B and supported by HPLC-ES-MS [not shown]), while the trace of 3-C13-LAS was converted largely to putative 3-C5-SPC (9.3 min in Fig. 5B). Similarly, the peaks attributed to C13-SPdCs (13.2 min), to C9-SPdCs (10.0 and 10.2 min), and to a lesser extent C7-SPdCs (7.9 min) and C5-SPdC (5.1 min) were reduced (Fig. 5B compared with Fig. 5A), because not all isomers were subject to two ω-oxygenations.

The logic behind the attribution of peaks was further strengthened by observations made with a preparation containing reduced amounts of 7-C13-LAS and 6-C13-LAS in 5-C13-LAS, 4-C13-LAS, 3-C13-LAS with a trace of 2-C13-LAS (Fig. 5C). The data from HPLC-ES-MS (not shown) confirm the low amounts of C9-SPC (13-5-13.6 min in Fig. 5C) and the corresponding

SPdCs (e.g. C5-SPdC at 5.1 min and C9-SPdC at 10.0 min in Fig. 5C). The major SPC was C7-SPC(s) with a significant contribution from C5-SPC(s) (data from HPLC-ES-MS, not shown); the corresponding SPC-2Hs and SPC+2Cs were also present. The corresponding separation with gradient system I (Fig. 5C) showed two C7-SPCs, the smaller peak (3-C7-SPC at 11.9 min in Fig. 5C) derived from 5-C13-LAS, and the larger peak (4-C7-SPC at 11.5 min in Fig. 5C) from the more abundant 4-C13-LAS. The C5-SPC is effectively 3-C5-SPC (9.3 min in Fig. 5C) from 3-C13-LAS. The relatively small peak of 3-C5-SPC, which arose from the most abundant LAS (Fig. 5C), could perhaps be explained by a large portion of the SPC+2C species (5-C7-SPC), which presumably co-eluted with 4-C7-SPC (at 11.5 min in Fig. 5C) to give an anomalously high peak.

Growth of strain DS-1^T in traces of 5-C13-LAS, 4-C13-LAS, 3-C13-LAS and mainly 2-C13-LAS yielded products which not only confirmed the pattern elucidated above but allowed the data to be confirmed by co-chromatography with known material. The peaks at about 13.6 min (Fig. 5D) testify to the tailing of 7-C13-LAS and 6-C13-LAS during semi-preparative HPLC. The peaks at 11.9 min (3-C7-SPC from 5-C13-LAS), 11.5 min (4-C7-SPC from 4-C13-LAS and 5-C7-SPC from 3-C13-LAS), and 9.3 min (3-C5-SPC from 3-C13-LAS) were discussed above. The major product detected by HPLC-ES-MS (not shown) was a C5-SPC which chromatographed at 9.2 min (Fig. 5D) and co-chromatographed with the 4-C5-SPC obtained from 2-C11-LAS in earlier work (Schleheck *et al.* 2003c). Comparatively large amounts of 4-C5-SPC-2H were generated (peak at 8.7 min, cf. Schleheck *et al.* 2003c); the identification of the peak was confirmed by the UV-spectrum, which was typical of SPC-2Hs (see above).

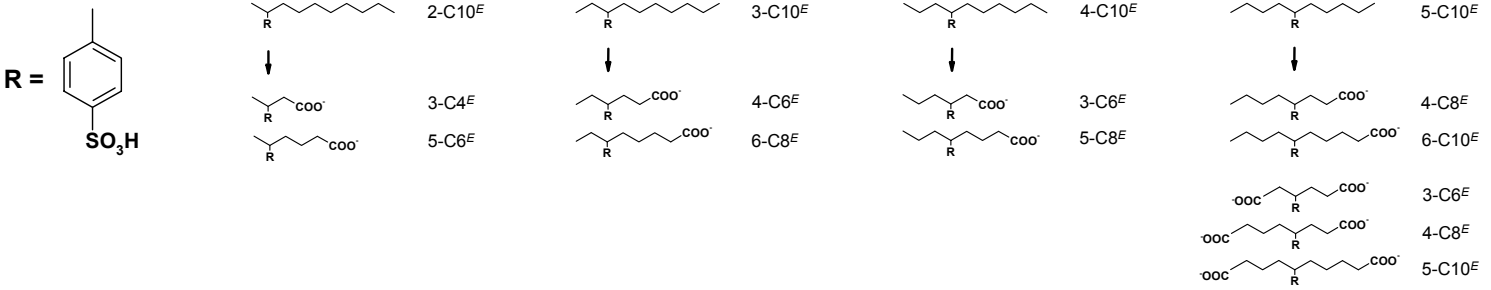
Conclusions

Much of the structure deduction was derived from logic and experience, aided by mass spectra, but with very few NMR data to confirm the positions of substituents. The NMR data of the long chains are not easy to interpret (Schleheck 2000), so the effort to obtain them from the µg-amounts of some of the chemicals used here would not be well invested. So the few cases of co-chromatography with authentic material played a key role in establishing the overall picture of degradation of LAS.

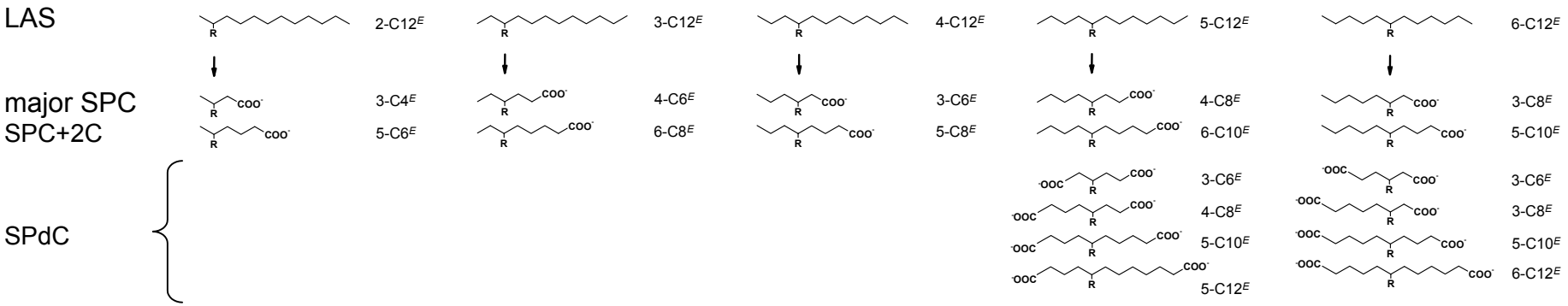
Fig. 7 illustrates the array of SP(d)Cs, which we showed (Eichhorn and Knepper 2002; Dong *et al.* 2003; Schleheck *et al.* 2003c; this paper) or infer to be excreted by strain DS-1^T during growth with commercial LAS. The attack is initiated by ω-oxygenation (a P450 system) and oxidation to the corresponding SPC, following the `distance principle´ (Swisher 1987). This SPC can be subject to β-oxidation, or to a second ω-oxygenation (if the sulfophenyl substituent is at the 5-, 6- or 7-position), or it can be excreted. β-Oxidation proceeds to within four, or a minimum of three, carbon atoms from the sulfophenyl substituent. If β-oxidation is active on only one of the two portions of the sidechain, two types of excretion product are possible, an SPC and an SPC-2H; these can obviously be excreted in any cycle (Dong *et al.* 2003), but the largest amounts excreted are usually the SPC, which cannot undergo a further round of β-oxidation, and the corresponding SPC-2H, with the SPC+2C. When β-oxidation is active on both portion of the sidechain, only SPdCs are excreted, but in this case, the SPdC, which cannot undergo further β-oxidation is the least common excretion product.

The number of compounds excreted is considerable. The 20 LAS congeners yield 11 major SPCs, 11 SPC-2Hs and 11 SPC+2Cs, most (>22) of them as enantiomer-pairs. The formation of 17 SPdCs can be inferred (Fig. 7), most (12) of them as enantiomer-pairs. Relatively few of these compounds seem to have been detected in the environment, whereas their disappearance

C10


R =
SO3H
2-C10E
3-C4E
5-C6E
3-C10E
4-C6E
6-C8E
4-C10E
3-C6E
5-C8E
5-C10E
4-C8E
6-C10E
3-C6E
4-C8E
5-C10E

C12


LAS
major SPC
SPC+2C
SPdC
2-C12E
3-C4E
5-C6E
3-C12E
4-C6E
6-C8E
4-C12E
3-C6E
5-C8E
5-C12E
4-C8E
6-C10E
3-C6E
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5-C10E
6-C12E

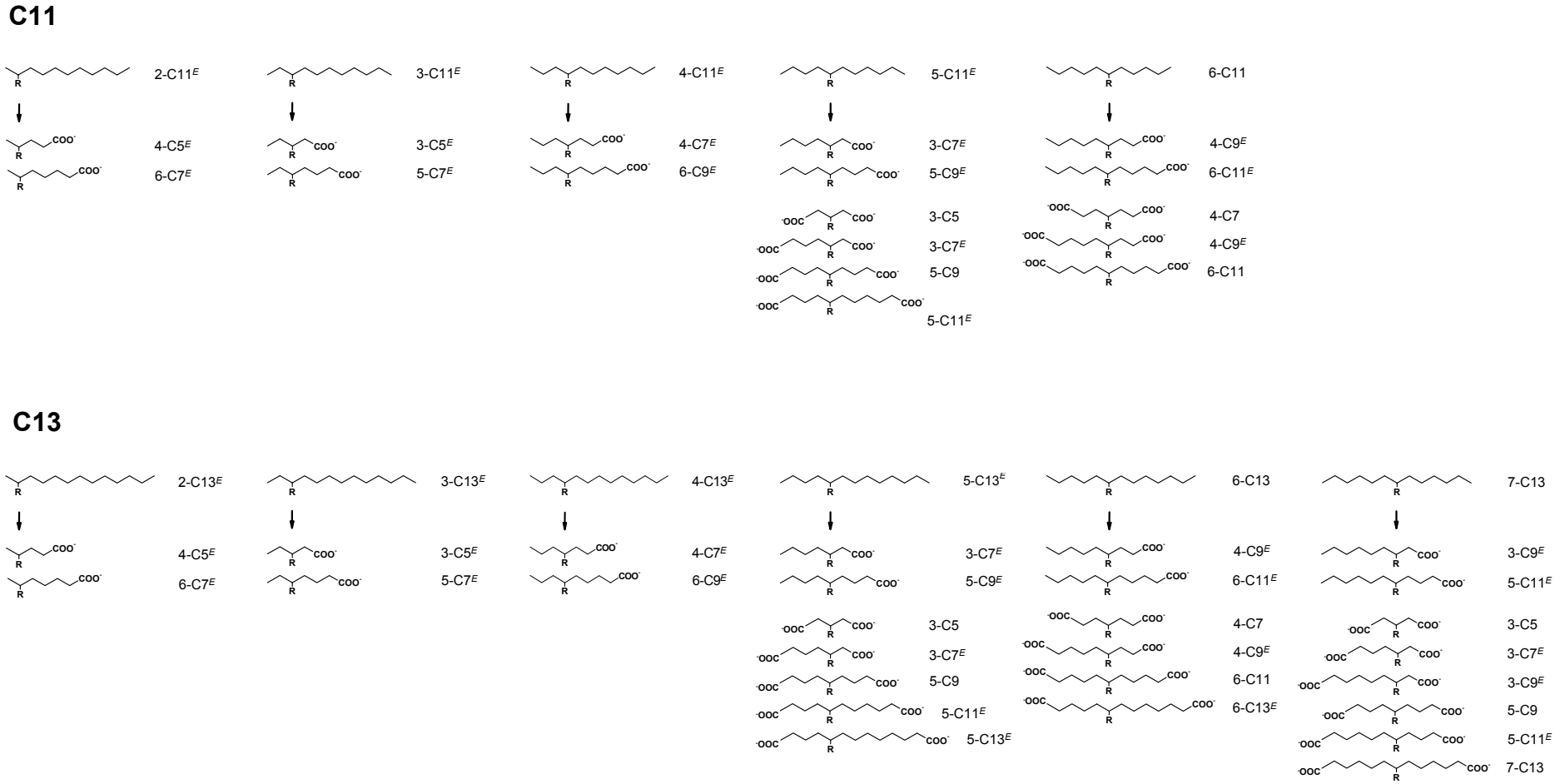

FIG. 7. Diagramatic representation of all LAS congeners with C-even (top page) and C-odd alkyl chain (this page), as present in commercial LAS, and the sets of SPC, or of SPC and SPdC, derived from their degradation by strain DS-1^{T}. The SPC-2H species derived from major SPC are not represented, and not products from presumed α-oxidation (Schleheck *et al.* 2003c). ^{E}, Racemic mixture.

in samples from many environments shows their degradation (Dong *et al*. 2003). SPCs are degraded by bacteria which, where known, have narrow substrate ranges (Schleheck *et al.* 2003c). It remains to be seen, how many organisms are needed to complement an organism like *P. lavamentivorans*^{T} in the completion of mineralization of LAS.

ACKNOWLEDGEMENTS

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CHAPTER 7

General Discussion

GENERAL

The mineralization of complex mixtures of complex surfactant molecules has been solved in native environments by microbial action in communities (Chapters 4 and 5). This is not the first surfactant-degrading community to be observed (e.g. Kroon and van Ginkel 2001), or even the first LAS-degrading community (Hrsák 1995a), but it is the first community to allow access to all aspects of a heterotrophic degradative pathway, that has confounded researchers for over 40 years. It may also open the way to a molecular understanding of the degradative pathways of the major commercial surfactants in household detergents.

The novel organism

The key to the thesis was the availability of a bacterium able to degrade LAS at high substrate concentrations (Schleheck *et al.* 2000). Its properties (Chapter 2 and Dong *et al.* 2003) are apparently such, that nobody could isolate it previously: it does not dominate the enrichment culture (using LAS as substrate; discussed in Dong *et al.* 2003), it requires a solid support, and it effectively does not grow with complex medium. The organism is apparently widespread (Dong *et al.* 2003), and other isolates with similar morphology, but with low resistance to toxicity can be obtained with a different enrichment technique (Schleheck, unpublished).

The novel organism now has a name, *P. lavamentivorans*^{T} (Chapter 2), and it must be possible to use molecular techniques to explore its occurrence in nature, e.g. phylogenetic staining with fluorescent oligonucleotides complementary to the 16S rRNA-gene sequence.

Toxicity and biofilms

One of the oldest functions of surfactants, from prehistory till to the present day, is to eliminate bacteria: we use surfactants to remove bacteria from dishes, clothes or floors, where

our ancestors used them to clean wounds. We recognise how effective modern surfactants can be in dissolving cell membranes, so effective surfactant degraders like *P. lavamentivorans*^{T} apparently use a biofilm to protect themselves (Chapters 2 and 5). Other heterotrophic LAS-degraders seem to have a much poorer resistance (Schleheck, unpublished). The nature of the inducible biofilm, its role in protecting the cell, and the nature of the regulation of its formation are now open to study.

Balancing specific and non-specific enzymes

The very different communities seen, or indicated in Chapters 4 and 5, all derive from the ability of *P. lavamentivorans*^{T} to catalyse ω-oxygenation at the terminal methyl terminus of *n*-alkyl groups. The range of acceptable substituents on this alkyl group is indicated in Table 1, Chapter 2: it is inferred that the reaction is always catalysed by the P450-monooxygenase detected in Chapter 3 (Table 1). The specificity of the monooxygenation reaction itself is complemented by a very broad substrate range, and the problems, that can be envisaged in the regulation of a system with this substrate range, are possibly balanced by its partly constitutive expression (Chapter 3). This in turn leads to the risk of reactive oxygen species being generated when not needed. Here, too, there is a range of topics to be explored.

The problem of substrate complexity in the degradation of LADPEDS (Chapter 4), where *P. lavamentivorans*^{T} obviously generates hundreds of intermediates (Chapter 4 and Chapter 6), is solved in the laboratory by altering the conditions to make the aromatic sulfonate group to the common factor for growth, namely the sole sulfur source. Here, the specific desulfonation of a wide range of substrates paves the way for renewed attacks in the carbon cycle (Chapter 4). The desulfonation reaction is presumed to be under the `sulfate-starvation induced´-stimulon within the *cysB*-controlled regulon, which is under study elsewhere (Kertesz 2000; Kertesz and Kahnert 2001). Data from work in industrial laboratories (Chapter 4) make it clear, that the three-step degradation of LADPEDS demonstrated in this laboratory (Chapter 4), also takes place in soil.

A different form of specificity can be observed in the degradation of SPCs by e.g. *C. testosteroni* KF-1 (Chapter 5), where the specificity of the organism, be it at the level of regulation, transport or catalytic enzyme, is so narrow, that a large community is needed to complete the catabolism initiated by strain DS-1^T.

Novel pathways and enzymes

The initial degradative pathway for LAS has been suggested for many years (e.g. Swisher 1970), and confirmed and expanded by the current series of experiments (Eichhorn and Knepper 2002; Dong *et al.* 2003, Chapters 4, 5, 6). One critical expansion is on the nature of the ω-oxygenation, which can now be examined in pure culture: perhaps a different strain of *P. lavamentivorans* will express an enzyme that is active in cell extracts, and thus be more convenient than strain DS-1^T (Chapter 3). Otherwise, the P450-typical absorption could be followed to allow protein purification (cf. Peterson *et al.* 1992).

A soluble ω-oxygenase system requires, that LAS, which is highly polar, must be transported into the cell. There must also be at least one transport system to export SPCs. Similarly, there must be transporters for SPCs to enter e.g. *C. testosteroni* KF-1 (Chapter 5), and for sulfate or sulfite to leave it. Our understanding of sulfonate transporters is still rudimentary (Mampel 2000; Kertesz 2001), so analyses of transporters should be coupled to characterisations of catabolic enzymes.

Even the `routine´ catabolic enzymes in *P. lavamentivorans* are unknown. What classes of alcohol and aldehyde dehydrogenases are involved in the generation of the SPC? What modification to `standard´ β-oxidation enzymes, which are often specific for certain chain lengths, is required to cope with the substrate range indicated in Chapter 2, Table 1?

The pathway of degradation of the SPCs, and presumably of the SPC-2Hs, can be seen to proceed via 4-sulfocatechol (Schulz *et al.* 2000; Dong *et al.* 2003, Chapter 5), with the possibility that 4-sulfophenol is also an intermediate in the degradation of 3-C4-SPC by *C.*

testosteroni spp. Preliminary data from a manuscript in cooperation with Dr. H.-P. E. Kohler confirm the transient excretion of 4-sulfophenol during the degradation of 3-C4-SPC by a different isolate, so the removal of the alkyl-side chain does yield 4-sulfophenol. There seems to be no similar reaction in the literature. If no isolate yields an active enzyme in cell-free extract, experience in this laboratory shows that *C. testosteroni* is amenable to transposon mutagenesis, as a tool to elucidating genes involved in degradative pathways (Tralau *et al.* 2003b).

The *ortho* cleavage pathway of 4-sulfocatechol is clearly indicated in Chapter 5, and there is solid evidence for this enzyme in the *C. testosteroni* spp. used. However, the later enzymes in the pathway, especially the desulfonation reaction, have not been studied with the rigour shown towards the ring cleavage (cf. Contzen *et al.* 2001).

The research (Chapter 5) also shows that 4-sulfophenol, whose degradation has been known for several decades (Thurnheer *et al.* 1986) but not examined, is also degraded via 4-sulfocatechol in *C. testosteroni*. It seems reasonable to postulate that 4-sulfophenol-2-mono-oxygenase in strain KF-1 will resemble the flavoprotein 4-hydroxybenzoate-2-monooxygenase (cf. Entsch and Ballou 1989; Seibold *et al.* 1996), and specific inhibitor of flavoprotein monooxygenase is available (cf. Chapter 3, Tomasi *et al.* 1995). Here too, should an approach via enzyme activity fail, one could resort to transposon mutagenesis to elucidate the genes involved in degradation.

The data in Chapter 2 indicate that the following surfactants will be subject to attack by ω-oxygenation and β-oxidation: primary alkylsulfonate, secondary alkylsulfonate, α-olefin-sulfonate, methylestersulfonate, N-alkyl-N-methyltaurate, alkylsarcoside, alkaneethoxylate, alkaneethoxylate sulfate, alkyldiglycolether sulfate, alkylglutamic acid, polyoxyethylene sorbitan monoalkanoate, alkyl poly-glucoside, and fatty-acid glucosamide. In the cases of primary docecanesulfonate and N-oleyl-N-methyltaurate, preliminary data indicate the formation of biodegradable products, putative 6-sulfohexanoate and an N-carboxylate of N-methyltaurine, respectively. The latter is degradable in pure culture (preliminary results from a practical class).

It seems likely that similar experiments can be done with each type of surfactant, which will make elucidation of degradative pathways for all classes of commercial surfactants a feasability. Very little detail is currently available on these pathways.

The degradative community for LAS

Degradative communities able to dissimilate LAS can be obtained from all local sewage works and all pristine samples examined (Dong *et al.* 2003, Dong unpublished). All behaved similarly (Dong *et al.* 2003), so a similar community structure is assumed. This structure may be considered as a linear sequence. *P. lavamentivorans* grew and excreted SPCs, whether or not SPC-degraders were present. The SPCs degraded by any one organism were unaffected by the presence of another degraded (Chapter 5, Fig. 2), so neither synergy nor antagonism was detected. The organisms were all prototrophic, so no exchange of metabolites amongst the community members was necessary. It remains to be seen, how large the community must be to catalyse the complete dissimilation of the LAS carbon.

APPENDIX

Testing the biodegradability of linear alkylbenzenesulfonate (LAS) surfactants and of their aerobic degradation intermediates under anoxic conditions

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2003, in preparation.

ABSTRACT

Linear alkylbenzenesulfonate surfactants (LAS) and their aerobic degradation intermediates, sulfophenylcarboxylates (SPC), can be found in large amounts in anaerobic sediments of e.g. rivers and marine bays, or in anaerobically digested sludge from sewage treatment plants, and increased the level of interest in the anaerobic dissimilation of LAS and SPC. So far, these processes are commonly reported not to occur. In this study, a further approach was set up to detect anaerobic dissimilation of LAS, or of SPC, using an fixed-bed reactor system running under strictly anoxic conditions. A mixture of biomass from various sources was used as inoculum, and the compounds were present as the sole added source of carbon and electrons in salts-medium with sulfate or nitrate as electron acceptor. The tested biomass showed no capacity to utilise LAS or SPC under these conditions in incubation periods of up to eight months. Not degraded by enrichments cultures under these conditions were also the hydroxyphenylcarboxylates (HPC), which we generated from SPC biologically through desulfonation.

INTRODUCTION

Linear alkylbenzenesulfonate (LAS, C10-C13) surfactant is quantitatively biodegraded under aerobic conditions as known for many years (Sawyer and Ryckman 1957, see also Swisher 1987; Schöberl 1989). Details on the microbial community and the degradative pathway(s) became only recently available (Dong *et al.* 2003; Schleheck *et al.* 2003c), through the isolation of a representative organism which catalyses the initial, aerobic degradation of LAS to mainly sulfophenylcarboxylates (SPC; Fig. 1), *Parvibaculum lavamentivorans*^{T} DS-1 (Schleheck *et al.* 2000; Schleheck *et al.* 2003e).

Interest in the anaerobic degradation of LAS is more recent. The discovery of LAS (and of SPC) in anaerobic sediments (Tabor and Barber 1996; Léon *et al.* 2000) and anaerobically digested sludge from wastewater treatment plants (up to 15 g/kg; Sanz *et al.* 2000), and political arguments in Denmark and other European countries (Elsgaard *et al.* 2001) newly raised the level of interest in the anaerobic degradation of LAS (UMSICHT 2003). Nonetheless, in 1992, all published claims for anaerobic degradation of LAS were considered to be invalid (Painter and Mosey 1992, see also Berna *et al.* 2001; UMSICHT 2003), but the anaerobic attack of LAS is principally possible, as shown when LAS was bioavailable as a source for sulfur under strictly anoxic conditions (Denger and Cook 1999). Furthermore, alkanes (and aromatic compounds) are degraded under anoxic conditions *via* addition of fumarate in a reaction best characterised for toluene (Heider *et al.* 1998; Ehrenreich *et al.* 2000; Rabus *et al.* 2001).

Continuous fixed-bed reactor systems have been proposed to study the anaerobic degradation of soluble chemicals or waste waters (e.g. Wagener and Schink 1987; Baumann and Mueller 1997). Our aerobic work with LAS emphasised the importance of surfaces for biodegradation of LAS (Schleheck *et al.* 2003e) and the advantage of using trickling filters to establish degradative cultures from diverse inocula in the laboratory before considering the organisms and the degradative reactions involved (Schleheck *et al.* 2000).

We thus aimed to detect anaerobic dissimilation of LAS and SPC in anoxic fixed-bed (AFB) reactor systems inoculated with biomass from various (anoxic) environments (sediments, sludge, soils). The compounds represented the sole added source of carbon and electrons in salts medium with sulfate or nitrate as electron acceptor (sulfate- or nitrate-reducing conditions). We intended to monitor the biodegradation by taking samples from the culture fluid at intervals, and HPLC analysis to determine LAS and/or SPC disappearance.

Desulfonated SPCs (HPC, 4-hydroxyphenylcarboxylates, Fig. 1), which were derived biologically from aerobic conversion of SPC by *Rhodococcus opacus* ISO-5 under sulfur-limited

growth conditions (see Schleheck *et al.* 2003d), were also tested for their biodegradability under these conditions.

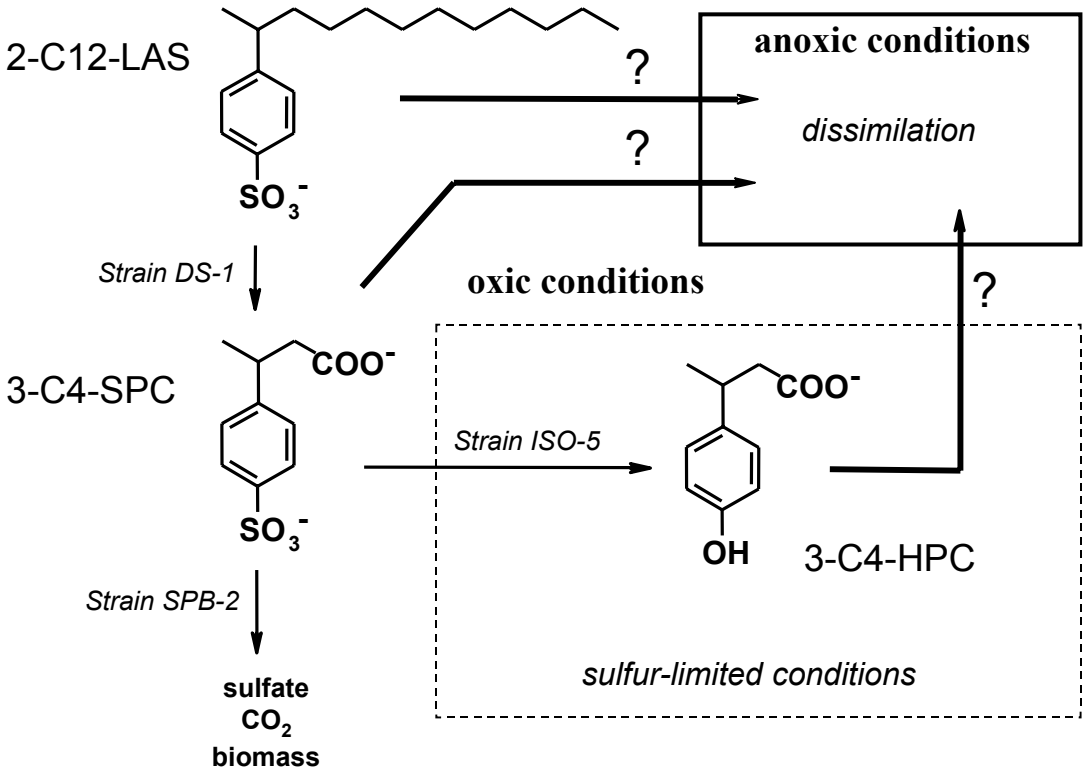

FIG. 1. Established LAS-degradative reactions in oxic environments and the questioned anaerobic dissimilation of LAS, or of aerobic degradation intermediates derived therefrom. The scetch shows 2-(4-sulfophenyl)dodecane (2-C12-LAS) as an example, which is degraded by *P. lavamentivorans*^{T} DS-1 mainly into 3-(4-sulfophenyl)butyrate (3-C4-SPC). 3-C4-SPC is mineralised aerobically (Strain SPB-2), and can also be desulfonated into presumed 3-(4-hydroxyphenyl)butyrate when utilised as source of sulfur by *R. opacus* ISO-5. Commercial LAS (C10-C13) was used in this work, and SPCs and HPCs derived therefrom (dominant side-chain length C4-C9).

MATERIAL AND METHODS

Anoxic fixed-bed reactor system

Two reactor systems were mounted as illustrated in figure 2, and incubated at room temperature (appr. 22 °C) in the dark. All glass pipes of the reactor systems were connected by fluor-rubber tubes (Tygon ISO-VERSINIC), and all openings were sealed by butyl-rubber stoppers; the tubing of the peristalic-pumps was of fluor-rubber material (Viton, inner diameter 1.5 mm, outer diameter 4 mm).

After setting up, the reactor was washed with 5 L destilled water (degassed under vacuum) for two days (2 ml/min), during which the gas phase was exchanged by fresh N_2/CO_2-gas (80:20 v/v, Lenogan) at intervals to remove atmospheric oxygen. The liquid phase was changed to appropriate anoxic culture medium (5 L, without additional carbon and electron source) and pumped through the reactor tube for additional two days (2 ml/min) (the gas phase was repeatedly exchanged).

During the inoculation procedure, the pump was shut off, and a constant flow of N_2/CO_2-gas was applied when the stoppers were opened: the anoxic suspension of biomass was slowly poured through a tube onto the first half of the polyester fleece, and a surplus of liquid drained in to the medium reservoir. After allowing the biomass to settle (1-2 days), the pumping was started (0.5 ml/ml), and after additional 10 - 15 days, the experiment was started by addition of LAS or SPC to the medium reservoir. The system was routinely monitored for substrate disappearance by taking samples from the liquid phase and HPLC analysis.

Materials

The linear alkylbenzenesulfonate (LAS) surfactant in use was commercial LAS (C10-C13) (Marlon A 350, Hüls). 4-Sulfophenylcarboxylates (SPCs, main chain length C4-C9) were derived after growth of *P. lavamentivorans*^{T} DS-1 with 1 mM LAS (Schleheck *et al.* 2000; Schleheck *et al.* 2003e). 4-Hydroxyphenylcarboxylates (HPCs) were derived after growth of *Rhodococcus opacus* ISO-5 with 50 µM SPCs under sulfur-limited growth conditions (Schleheck *et al.* 2003d), and extracted from the culture medium after conversion by semi-preparative HPLC (Schleheck *et al.* 2003d). The general chemicals of high purity were purchased from Fluka, Merck, or Sigma-Aldrich. Gases were obtained from Messer Griesheim (Ludwigshafen, Germany).

Inocula for the reactor systems were from an upflow-anaerobic sludge bed reactor (University of Madrid, Spain), from anoxic, marine sediment of the Bay of Cadiz (Spain), from anoxic, freshwater sediment of Lake Constance (Wallhausen, Germany), from oxic and anoxic digested sludge from the industrial and local sewage treatment plants in Ludwigshafen (BASF), Konstanz, and Radolfzell (each in Germany), and

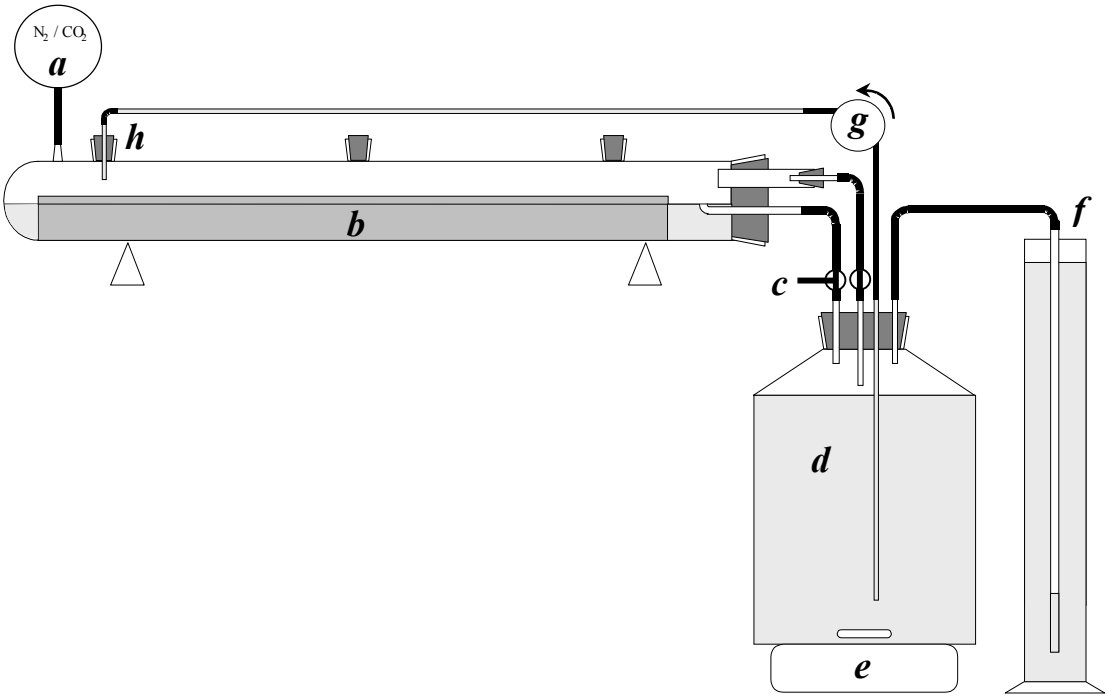

FIG. 2. Anoxic fixed-bed (AFB) reactor system. Biomass settled on polyester fleece in a horizontal glass tube was suspended in anoxic culture medium (N_2/CO_2-gas phase), which was supplied from a medium reservoir (N_2/CO_2-gas phase) into one end of the tube. After passage through the polyester fleece, the culture medium drained into the medium reservoir (circular flow, 0.5 ml/min). The reactor system was kept under N_2/CO_2-overpressure by the back-pressure vessel (outlet-valves opened); loss of pressure was compensated by addition of fresh N_2/CO_2-gas, if appropriate. During inoculation (see Material and Methods), or when samples of culture medium and of biomass were taken from the reactor tube through the openings, the gas phase in the reactor tube was extensively flushed with N_2/CO_2-gas (outlet-valves closed), and several minutes afterwards (outlet-valves opened). After exchange of the medium reservoir (outlet-valves closed), the gas phase in the reservoir was flushed with N_2/CO_2-gas (outlet-valves opened). A sample of liquid from the outlet of the reactor tube was routinely taken when drained through the sample-outlet under a flush of N_2/CO_2-gas. (*a*), N_2/CO_2-gas inlet; (*b*), reactor tube with polyester fleece (N_2/CO_2-gas phase); (*c*) outlet-valves and sample outlet; (*d*), medium reservoir (N_2/CO_2-gas phase); (*e*), magnetic stirrer; (*f*), back-pressure vessel; (*g*), peristaltic pump; (*h*), stopper with medium-inlet. Dimensions: reactor tube, 50 cm in length and 6 cm in diameter, 1 L in liquid volume; polyester fleece, 40 cm in length, 6 cm in broadness, 3 cm in height; culture medium reservoir, 5 L in volume; back-pressure vessel, 40 cm in height.

from alkane-contaminated loamy soil (Stockach, Germany), and were stored at 4 °C under a N_2-atmosphere. Samples from these inocula (in total appr. 100 g wet-weight) were suspended in anoxic salts medium (0.3 L), and applied to the polyester fleece (see above).

Culture media and growth conditions

Dissimilation tests under nitrate-reducing conditions were done with $NaHCO_3$-buffered (30 mM), mineral salts medium (Denger and Cook 1997) supplemented with 1 % NaCl and 10 mM $NaNO_3$, and was prepared anoxically (Widdel and Pfennig 1981); Na_2S was added (0.2 mM). The medium for dissimilation tests under sulfate-reducing conditions contained 7 mM Na_2SO_4 instead of $NaNO_3$, and the reducing agent titanium(III)nitrilotriacetate (0.5 mM). The redox indicator was resazurine (1 mg/L). LAS and SPCs were added in concentrations as stated in the Results.

Enrichment cultures (10 ml in 25-ml glass bottles) with HPCs as sole added source of carbon and electrons were inoculated with mixed biomass (see above, 1 ml), sealed with butyl-rubber stoppers (N_2/CO_2-gas phase), and routinely cultivated at 30 °C in the dark.

Parvibaculum lavamentivorans DS-1 (DSM 13023^T, NCIMB 13966^T) and *Rhodococcus opacus* ISO-5 (DSM 44600) were available in the laboratory and grown under conditions as described previously (Schleheck *et al.* 2003d; Schleheck *et al.* 2003e).

Analytical methods

LAS, SPC and HPC was analyzed by HPLC as given elsewhere (Schleheck *et al.* 2000), and nitrate and sulfate concentrations were determined by ion-chromatography with suppression (Laue *et al.* 1996). The apparent redox potential of the culture medium was determined by a platinum electrode and a calomel-reference electrode (Ebert and Brune 1997) calibrated with saturated solutions of quinhydrone in standard pH-buffers (benzoquinone/hydroquinone redox-pair at pH 4-7) (Bohn 1971).

RESULTS

Incubation of sediments in the AFB-reactor running under sulfate-reducing conditions with LAS or SPC as sole added source of carbon and electrons.

The mixture of biomass was added to AFB reactors running under sulfate-reducing conditions. During the first 10 days after inoculation, and before the substrate was added to the medium reservoir (LAS or SPC, see below), the turbidity of the liquid in the reservoir only slightly increased, as detectable by eye. This indicated growth of some organisms, which was confirmed when biomass was collected from samples by centrifugation and observed under the microscope (various morphotypes). Dissimilatory sulfate reduction was indicated by gas production detected in the back-pressure vessels (bubbles, smell), by precipitation of FeS, and by the consumption of sulfate (not shown). The reactor systems were strictly anoxic throughout the whole incubation times (up to eight month, see below) as verified by the black colour of the sediments (FeS), an colourless redox-indicator, and by the apparent redox potential of the liquid phase determined at values below –290 mV.

At day 10 after inoculation, LAS was added to the reservoir of one reactor to give a final concentration of 100 µM in the medium reservoir. The LAS concentration analysed in samples from the reactor-outlet stabilised to about 80 µM LAS (total peak area $A_{220\ nm}$) within two days (Fig. 3). This apparent loss of LAS could be attributed almost solely to the dilution effect since 1 L LAS-free salts medium remained in the reactor tube when LAS was added to the reservoir. This indicated that the amount of LAS sorbed to e.g. biomass and polyester fleece (cf. Schleheck *et al.* 2003e) was negligible under these conditions. During ten weeks of incubation, no further decrease of LAS was measurable (Fig. 3), and no increase of turbidity was detectable in the reservoir. The microorganisms were active as indicated by gas production. Comparisons of the HPLC chromatograms obtained from analysis of samples taken at begin and during the

experiment, and at the medium-inlet and outlet, showed all peaks of LAS principally unchanged (not shown). No new peaks indicative of possible products from a conversion of LAS appeared. We concluded that no degradation of LAS took place under the used conditions, and the experiment was stopped to change the reactor system to incubation of biomass under nitrate-reducing conditions (see below).

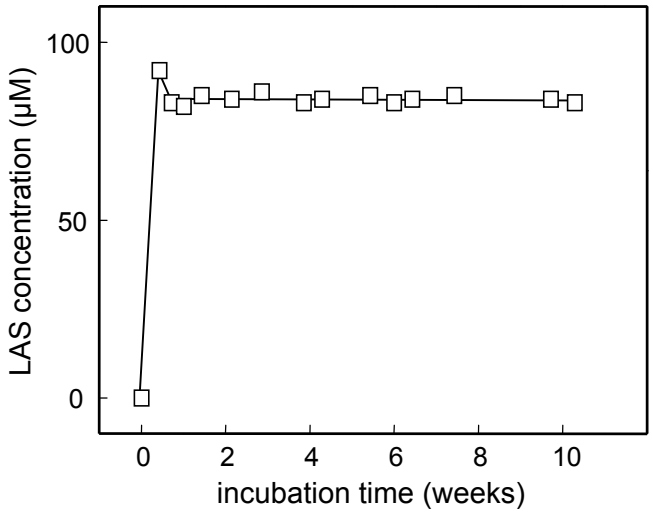

FIG. 3. LAS concentrations determined in the culture medium of the AFB reactor during incubation under sulfate-reducing conditions. The incubation was started by addition of LAS to the medium-reservoir, and samples were taken from the reactor-outlet at intervals and analysed by HPLC. The concentration was determined as total-peak area of the many peaks from commercial LAS (Fig. 4).

SPC was added to the medium reservoir of the second reactor at day 10 after inoculation to a final concentration of 60 µM. The SPC concentration stabilised at about 50 µM within about two days according to the dilution effect as described above. The reactor was incubated for eight months. HPLC chromatograms of samples taken at begin and at the end of the experiment were principally unchanged (Fig. 4), and no appearance of new peaks, representing possible products, was detected. No significant increase of turbidity in the medium reservoir was observed. We thus concluded that degradation of SPC, if any, was negligible under these conditions.

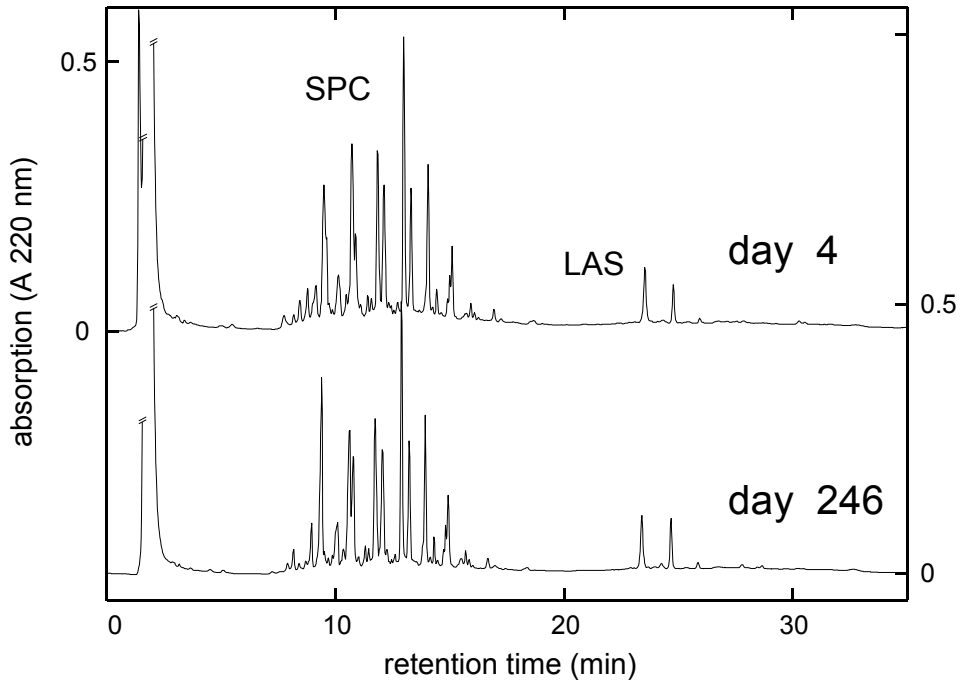

FIG. 4. HPLC chromatograms showing the levels of SPC at begin and at the end of incubation of the AFB reactor running under sulfate-reducing conditions. Samples were taken from the medium reservoir. Traces of LAS as residual from the degradation by *P. lavamentivorans*^{T} DS-1 were also present in the preparation.

Incubation of biomass in the AFB-reactor running under nitrate-reducing conditions with LAS and SPC as sole added source of carbon and electrons

The apparent redox potential of the salts medium in the reactor tube was determined at about –70 mV, thus indicating reducing conditions, and the reactor was inoculated with the mixture of biomass. The activity of the microorganisms in the sediment was indicated by the apparent consumption of nitrate and transient formation of nitrite as determined in samples taken from the reactor-inlet and outlet (not shown), and by a slight increase of turbidity in the medium reservoir (various morphotypes). At day 15 after inoculation, the experiment was started by addition of LAS to the medium reservoir to a final concentration of 100 µM LAS.

The concentration determined at the medium outlet stabilised to about 80 µM LAS within about two days (dilution), and ongoing decrease of LAS concomitant with the formation of presumed degradation intermediates was detectable in the following incubation period (Fig. 5). This apparent `anaerobic biodegradation´ was found to take place when traces of atmospheric oxygen entered the system through defective tubing at the peristaltic pump, and thus represented aerobic degradation of LAS into SPC: chromatograms of samples taken at the same time interval from the reactor-inlet and -outlet were virtually identical, but LAS was disappeared and products were formed when compared to the chromatogram of a sample from the reservoir (data not shown). Furthermore, the HPLC-peaks of presumed products (Fig. 5) cochromatographed with SPC derived from aerobic LAS-degradation (cf. Fig. 4), and these peaks showed identical UV-spectra compared to that of SPC and LAS (not shown). The defective tubing was exchanged, and no further disappearance of LAS or of SPC was observed during an incubation time of 3 month (data not shown), and after which the experiment was stopped.

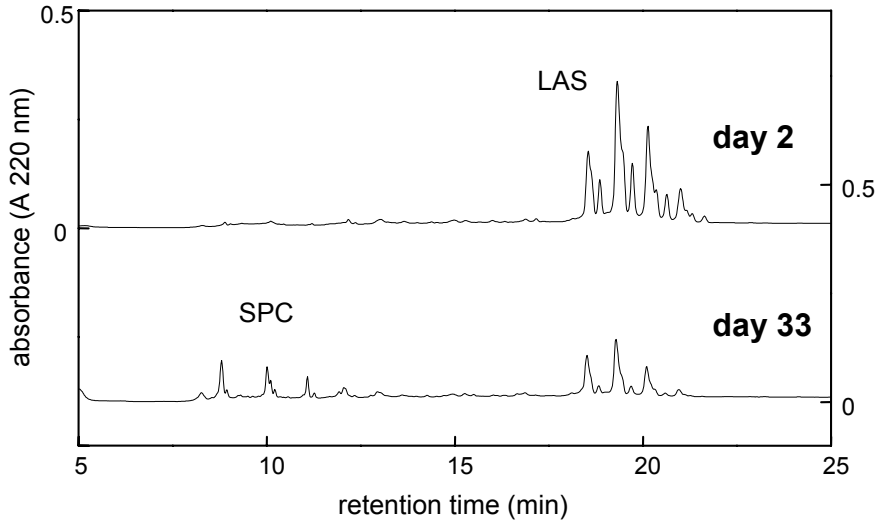

FIG. 5. HPLC chromatograms showing the levels of LAS and of presumed SPC at begin and at the end of the incubation period during which atmospheric oxygen entered the system.
Samples were taken from the reactor inlet.

Degradation test for the utilisation of HPC under anoxic conditions.

HPCs derived from SPCs (see Material and Methods) were present at concentrations of about 0.5 mM under sulfate- and nitrate-reducing conditions in salts-medium, which was inoculated with the mixture of biomass. Samples were taken at intervals and analysed by HPLC.

HPCs were not degraded in the sulfate-reducing enrichment during a three-months observation period. Chromatograms from the nitrate-reducing enrichment showed a completely different pattern of peaks, which we attributed to a spontaneous transformation of HPCs into nitrophenolic compounds by the presence of 10 mM $NaNO_3$ in salts medium. These compounds were not subject to microbial attack under these conditions during a three-months observation period.

DISCUSSION

It was shown that 15 – 20 % of the LAS in the influent sewage into a treatment plant is transferred with the sludge into the anaerobic digester. Though at best at low degree, removal of LAS in anaerobic digesters was monitored in field studies, ranging from 0 – 35 % (Berna *et al.* 1989; Giger *et al.* 1987), and in individual laboratory studies (e.g. 14 - 25%, Angelidaki *et al.* 2000), but it is yet not clear to which processes these removals can be ascribed (anaerobic dissimilation, binding, co-metabolism, anaerobic desulfonation).

The tested biomass from various environments showed in our experiments no potential to utilise LAS, SPC or HPC as sole source for carbon and electrons under strictly anoxic conditions, when either sulfate or nitrate were supplied as electron acceptor, and under the conditions we used. We thus contributed to a long line of laboratory experiments which failed to detect an anaerobic dissimilation of LAS (see Painter and Mosey 1992; Berna *et al.* 2001). The

anaerobic degradability of SPC and HPC derived from LAS by characterised aerobic degradation (Dong *et al.* 2003; Schleheck *et al.* 2003d) was tested here for the first time.

Administrative regulations in Denmark and other European countries (Elsgaard *et al.* 2001) concern the disposal of anaerobically digested sludge to land as a fertilizer, thus exposing the plant and soil life to LAS and SPC, which can be inhibitory to biological activity important for soil fertility and nutrient cycling (Elsgaard *et al.* 2001). Nevertheless, soils are usually aerobic systems (Kaspar and Tiedje 1982), and the microorganisms in soil degrade LAS and SPC efficiently (e.g. Waters *et al.* 1989; see also Dong *et al.* 2003; UMSICHT 2003).

We confirmed that the presence of molecular oxygen is the limiting parameter for the degradation of LAS. How easily LAS is degraded if molecular oxygen is available to the organisms was visible (see Fig. 5) when a defective tubing allowed atmospheric oxygen to enter the system: LAS disappeared steadily, and presumed SPCs were formed though in little amounts, and we thus conclude that also aerobic degradation of SPC took place in the tubing. The oxygen was apparently depleted when the liquid entered the reactor tube, since no further degradation was detectable in the reactor tube, and when the apparent redox potential determined in the liquid of the reactor tube still indicated anoxic conditions. The degradation processes stopped when the defective tubing was exchanged, and the system was oxygen-tight.

The `surface-active´ surfactant LAS can sorbe to solids e.g. the sediments under test, to the biomass, and to (other) surfaces being in sur-face to the liquid, such as to the glass ware or tubings, or to polyester fleece (Schleheck *et al.* 2003e). Initial fate of LAS observed in culture fluid is thus difficult to attribute to biodegradation (see Angelidaki *et al.* 2000): the steady fate of LAS up to ultimate disappearance during ongoing incubation is an evidence supporting biodegradation rather than sorption of LAS, whereas sorptive fate of LAS usually ends at a certain equilibrium determined by the test conditions (e.g. LAS concentration, sorbing-capacity and amount of sediment, water hardness). Further evidence supporting biodegradation would be the formation of degradation products, and the growth of microorganisms, i.e. the formation of

biomass coupled to substrate degradation. The latter is difficult to determine when e.g. suspensions of sludge are used in the test system (e.g. Angelidaki *et al.* 2000).

The constructed AFB reactor system was capable to maintain strictly anoxic conditions for long incubation periods (when allowing for the weak-point to be the tubing of the peristaltic pump). We intended to minimize the sorptive fate of LAS in the reactor through the use of a large reservoir of culture medium relative to the amount of biomass settled on the polyester fleece, and when the medium from the reservoir was circulated through the polyester fleece. A steady decrease of LAS would have indicated biodegradation. We hoped that growing organisms would soon be found to grow also in the medium reservoir when washed from the sediment during incubation, thus supporting a first enrichment of degradative organisms.

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