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The Missing Link in Linear Alkylbenzenesulfonate Surfactant Degradation: 4-Sulfoacetophenone as a Transient Intermediate in the Degradation of 3-(4-Sulfophenyl)Butyrate by *Comamonas testosteroni* KF-1▽

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Biodegradation of the laundry surfactant linear alkylbenzenesulfonate (LAS) involves complex bacterial communities. The known heterotrophic community has two tiers. First, all LAS congeners are oxygenated and oxidized to about 50 sulfophenylcarboxylates (SPC). Second, the SPCs are mineralized. *Comamonas testosteroni* KF-1 mineralizes 3-(4-sulfophenyl)butyrate (3-C4-SPC). During growth of strain KF-1 with 3-C4-SPC, two transient intermediates were detected in the culture medium. One intermediate was identified as 4-sulfoacetophenone (SAP) (4-acetylbenzenesulfonate) by nuclear magnetic resonance (NMR). The other was 4-sulfophenol (SP). This information allowed us to postulate a degradation pathway that comprises the removal of an acetyl moiety from (derivatized) 3-C4-SPC, followed by a Baeyer-Villiger monooxygenation of SAP and subsequent ester cleavage to yield SP. Inducible NADPH-dependent SAP-oxygenase was detected in crude extracts of strain KF-1. The enzyme reaction involved transient formation of 4-sulfophenol acetate (SPAc), which was completely hydrolyzed to SP and acetate. SP was subject to NADH-dependent oxygenation in crude extract, and 4-sulfocatechol (SC) was subject to oxygenolytic ring cleavage. The first complete degradative pathway for an SPC can now be depicted with 3-C4-SPC: transport, ligation to a coenzyme A (CoA) ester, and manipulation to allow abstraction of acetyl-CoA to yield SAP, Baeyer-Villiger monooxygenation to SPAc, hydrolysis of the ester to acetate and SP, monooxygenation of SP to SC, the *ortho* ring-cleavage pathway with desulfonation, and sulfite oxidation.

Linear alkylbenzenesulfonate (LAS) is the major synthetic laundry surfactant worldwide, with an annual production of 2.5×10^6 tonnes, which in Germany means 3 g of LAS per person and day, or about 4% of the carbon entering the sewage works (e.g., see reference 15); hence, its degradation is important. European LAS surfactant is nominally a mixture of 20 congeners, each of which is a linear alkane (C_{10}-C_{13}) subterminally substituted with a 4-sulfophenyl moiety (15) (Fig. 1); 18 of these congeners are chiral.

Mineralization of LAS has been known for 50 years (25), and the involvement of sulfophenylcarboxylates (SPCs) (Fig. 1) as intermediates in that process has been known for about 40 years (33). Recognition that the overall degradation of LAS involved communities of microorganisms developed 10 to 15 years ago (36), when routine high-performance liquid chromatography (HPLC) analysis of LAS and SPC in biological samples became available (16, 20). The coupling of HPLC technology to mass spectrometers has allowed the analysis of the transient SPC intermediates to be further improved (e.g., see references 7 and 19), such that we now have a comprehensive picture of some 50 SPC-like products (mostly chiral) formed from commercial LAS by the first-tier organism in an LAS-degrading bacterial community (27).

A heterotrophic, bacterial, LAS-degrading community comprises two tiers. The first tier of organisms, which is so far represented only by *Parvibaculum lavamentivorans* DS-1 (6, 12, 27–29), converts all LAS congeners to about 50 SPCs (and related compounds, see below) and cell material derived from the acetyl coenzyme A (acetyl-CoA) released through β-oxidation from the alkane moiety. In principle, each LAS congener yields three products that are released by strain DS-1, an SPC, an α,β-unsaturated SPC (SPC-2H), and an SPC from the previous round of β-oxidation (SPC+2C) (Fig. 1) (27, 28); sulfophenyl-di-carboxylates (SPdCs) are generated by β-oxidation of both ends of the alkane chain, e.g., from the "centrally substituted" 4- and 5-C10-LAS congeners (Fig. 1) (27), but these products are not relevant in this work. The second tier of organisms degrades these SPCs (and SPC-2Hs and SPdCs) to cell material, CO_2, water, and sulfate (28). In this tier, many different organisms must be active because all known representatives have a narrow substrate spectrum of only 3 to 4 individual SPC-like compounds (28, 30; Results). The present work centers on *Comamonas testosteroni* KF-1, which utilizes four known SPC-like compounds (Fig. 1), 3-(4-sulfophenyl)butyrate (3-C4-SPC), 3-(4-sulfophenyl)-Δ2-enoylbutyrate (3-C4-SPC-2H), 3-(4-sulfophenyl)pentanoate (3-C5-SPC), and 3-(4-sulfophenyl)-Δ2-enoylpentanoate (3-C5-SPC-2H). This work is focused on the metabolism of 3-C4-SPC. Strain KF-1 utilizes

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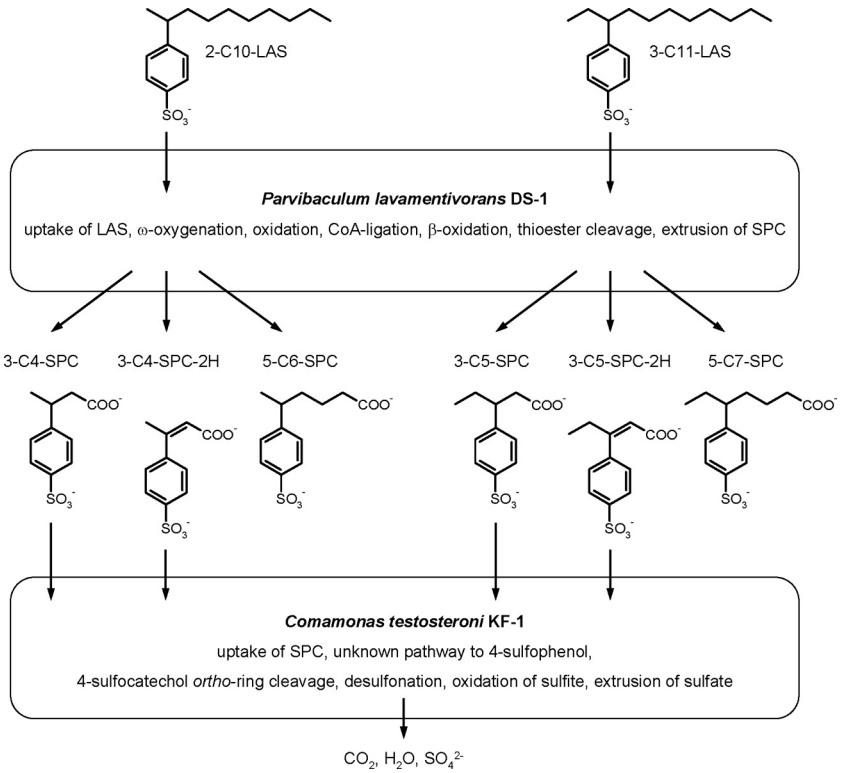

FIG. 1. Flow diagram of the primary degradation by *P. lavamentivorans* DS-1 of two LAS congeners to six SPCs, four of which are mineralized by *C. testosteroni* KF-1. None of the reactions or pathways indicated in strain DS-1 has been observed directly. None of the reactions in strain KF-1 has been observed directly, though 4-sulfophenol is a growth substrate (28): the major unknown in SPC degradation is the manipulation of the side chain.

both the (*R*)- and (*S*)-enantiomers of 3-C4-SPC (28), and this degradation may be enantioselective, resulting in different reaction rates (22), as observed for degradation of (*R*,*S*)-2-C4-SPC and (*R*,*S*)-4-C6-SPC by isolated *Delftia acidovorans* strains (28, 30).

We now report that *C. testosteroni* KF-1 utilized 3-C4-SPC with transient excretion of two degradation intermediates, whose identification allowed us for the first time to draft a complete degradative pathway for an SPC.

MATERIALS AND METHODS

Bacteria and growth media. *C. testosteroni* KF-1 (DSM 14576) (28) is available at the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). A phosphate-buffered, carbon-limited, mineral salts medium (35) supplemented with the appropriate carbon source was used. Cultures were incubated in glass tubes (Corning) in the 3-ml scale or in Erlenmeyer flasks in the 0.1- to 5-liter scale on a shaker at 30°C. Cultures were inoculated (1%) with outgrown, homologous preculture. Most carbon substrates were heat stable, and they were added to the salts medium before autoclaving; the exception was 4-sulfophenol acetate, which was filter sterilized. To effect oxygen limitation (see Results), a 0.1-liter culture of *C. testosteroni* KF-1 was grown with 9 mM 3-C4-SPC in a 0.3-liter Erlenmeyer flask at moderate agitation (140 rpm shaking), and samples of culture supernatant were analyzed for the appearance of novel peaks on HPLC chromatograms.

Chemicals. Racemic 3-C4-SPC was synthesized as described previously (28). Authentic 4-sulfoacetophenone (IUPAC name: 4-acetylbenzenesulfonate) was purchased from ABCR (Karlsruhe, Germany), and 4-sulfophenol acetate (IUPAC name: 1-phenol-4-sulfonate-acetate) was purchased from SYNCHEM (Felsberg-Altenburg, Germany). Standard chemicals were purchased from Sigma, Fluka, or Merck. Biochemicals (NADH, NADPH, NAD^+, and $NADP^+$) were from Biomol (Hamburg, Germany).

Preparation of cell extracts and protein separation. Each cell pellet (3 to 4 g wet weight) was resuspended in 3 to 4 ml of 50 mM Tris-H_2SO_4 buffer (pH 8.0) containing 0.1 mg DNase I (Sigma), and cells were disrupted by four passages through a French pressure cell (140 MPa, 4°C) (Aminco, Silver Spring, MD). Whole cells and debris were removed by centrifugation (17,000 × *g*, 20 min, 4°C) to obtain crude extract, and membranes were removed from crude extract by ultracentrifugation (370,000 × *g*, 30 min, 4°C), to obtain soluble protein extract. Fast protein liquid chromatography (FPLC) of soluble protein extract (up to 5 ml) was done on a MonoQ HR 10/10 column (Pharmacia) equilibrated with Tris-H_2SO_4 buffer (pH 8.0) at a flow rate of 1 ml/min, bound proteins were eluted from the column by a linear $NaSO_4$ gradient (to 0.2 M in 55 min and to 0.5 M in 10 min), and fractions (2 ml) were collected.

Enzyme assay. Oxygen uptake of cell suspensions or in cell extracts was measured in 50 mM Tris-HCl (pH 8.0) at 30°C in a Clark-type oxygen electrode (26). The aromatic substrate was present at 1 mM, and if appropriate, electron donor NAD(P)H was present at 1 mM. The 4-sulfoacetophenone or 4-sulfophenol-oxygenase activities were also determined photometrically as decrease of absorbance of NADPH or NADH, respectively. The 4-sulfophenol acetate-esterase activity was measured photometrically as increase of absorption of the reaction product 4-sulfophenol at 285 nm after addition of 1 mM 4-sulfophenol acetate.

Analytical methods. LAS and SPCs were routinely analyzed by reversed-phase high-performance liquid chromatography (HPLC) coupled to a diode array detector, using gradient system I (26) and a Nucleosil C_{18} column (125 by 3 mm; particle size, 5 μm [Knauer, Berlin]). HPLC for the enantioselective separation of (*R*)- and (*S*)-3-C4-SPC (gradient system II) was done using an Nucleodex-α-pm column (200 × 4 mm; particle size, 5 μm [Macherey-Nagel, Düren, Germany]) with a mobile phase as described earlier (31); the enantiomers eluted with baseline separation after 7.0 and 7.8 min, respectively. HPLC for the separation of 3-C4-SPC (retention time, 12.0 min), 4-sulfoacetophenone (10.5 min), NADPH (6.7 min), $NADP^+$ (8.1 min), 4-sulfophenol acetate (11.2 min), 4-sulfophenol (3.5 min), and 4-sulfocatechol (2.9 min) was done using gradient system III (mobile phase A, 50 mM potassium phosphate buffer [pH 2.2]; eluent

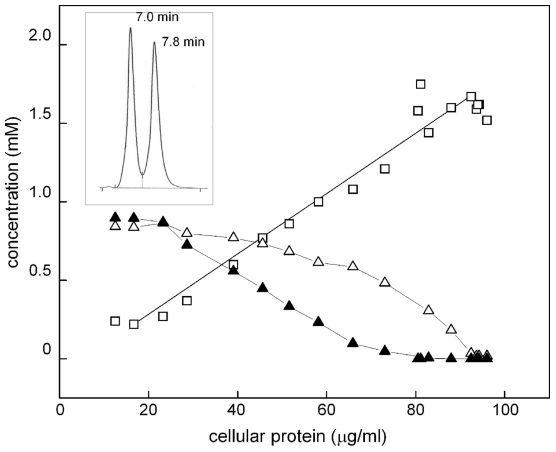

FIG. 2. Plot of (*R*)- and (*S*)-3-C4-SPC and sulfate concentrations versus cellular protein concentration during well-aerated growth of *C. testosteroni* KF-1 in 3-C4-SPC salts medium. The enantiomeric HPLC separation of (*R*)- and (*S*)-3-C4-SPC is given as an inset; no authentic standards of neither (*R*)- nor (*S*)-3-C4-SPC were available to assign identities to these peaks. Symbols: □, sulfate; △ and ▲, (*R*,*S*)-3-C4-SPC.

B, 100% methanol; flow rate, 0.5 ml/min) on a Nucleosil C_{18} column (see above). The gradient program was 100% A for 2 min, to 15% B in 3 min, to 80% B in 2 min, to 80% B for 7 min, and re-equilibration. Peaks in culture supernatant or in cell extract were identified in comparison with authentic 4-sulfoacetophenone, 4-sulfophenol acetate, 4-sulfophenol, or 4-sulfocatechol, based on (i) identical retention time, (ii) identical UV-visible spectrum, and (iii) cochromatography of peaks in samples that were spiked with an appropriate amount of authentic standard.

^{1}H nuclear magnetic resonance (NMR) and ^{13}C NMR spectra were obtained at 400.13 (100.61) MHz on a Bruker AV-400 NMR spectrometer. The ^{1}H and ^{13}C NMR spectra and the ^{1}H, ^{13}C two-dimensional (2D) correlation experiments were recorded with the Bruker standard pulse programs and parameter sets, and the ^{1}H/^{13}C chemical shifts were referenced internally using the resonance signals of CD_3OD at 3.31/49.0 ppm. The observed chemical shifts for 4-sulfoacetophenone (see Results), δ^1H (in ppm) with number of protons and assignment were 8.05 (2H, H-3), 7.93 (2H, H-2), 2.63 (3H, H-6), and δ^{13}C (in ppm), and with multiplicity and assignment, they were 199.5 (s, C-5), 150.6 (s, C-1), 139.4 (s, C-4), 129.5 (d, C-3), 127.2 (d, C-2), and 26.9 (q, C-6).

Acetate, as acetic acid, was determined by gas chromatography with a flame-ionization detector (17). Sulfate was determined turbidimetrically (32) or by ion chromatography (18). Total protein was determined following a Lowry-based protocol (14) with bovine serum albumin (BSA) as the standard.

RESULTS

Growth of *C. testosteroni* KF-1 with 3-C4-SPC. Strain KF-1 grew quantitatively with 3-C4-SPC in fully-aerated medium (Fig. 2), and sulfate was formed quantitatively (1.8 mM) from the (*R*)-3-C4-SPC and (*S*)-3-C4-SPC enantiomers initially present (0.9 mM each). Enantioselective HPLC showed that one of the enantiomers was used preferentially, though not exclusively (Fig. 2). After this enantiomer was exhausted, the remainder (about 40%) of the other enantiomer was utilized quantitatively. The specific growth rate (μ) was 0.09 h^{-1} and the molar growth yield was 5 g protein mol carbon^{-1}, which resulted in a maximal specific degradation rate of 4.9 mkat kg protein^{-1} for 3-C4-SPC.

Identification of intermediates excreted during 3-C4-SPC degradation. To stress strain KF-1 to excrete intermediates, the organism was grown with suboptimal aeration, as experienced elsewhere (30). The organism started to grow slower at high cell densities (Fig. 3A), after the substrate had disappeared but only half the anticipated sulfate had been released (e.g., at an incubation time of 25 h). This indicated that sulfur-containing metabolites had to be present in the growth medium. Indeed, HPLC analysis showed that two unknown compounds were excreted while 3-C4-SPC was utilized (Fig. 3B). After 3-C4-SPC was exhausted, the concentrations of the unknown compounds decreased while the concentration of sulfate further increased (Fig. 3A). Subsequent quantification of the compounds by HPLC (see below) closed the mass balance for sulfur throughout the growth experiment (cf. Fig. 3A and B).

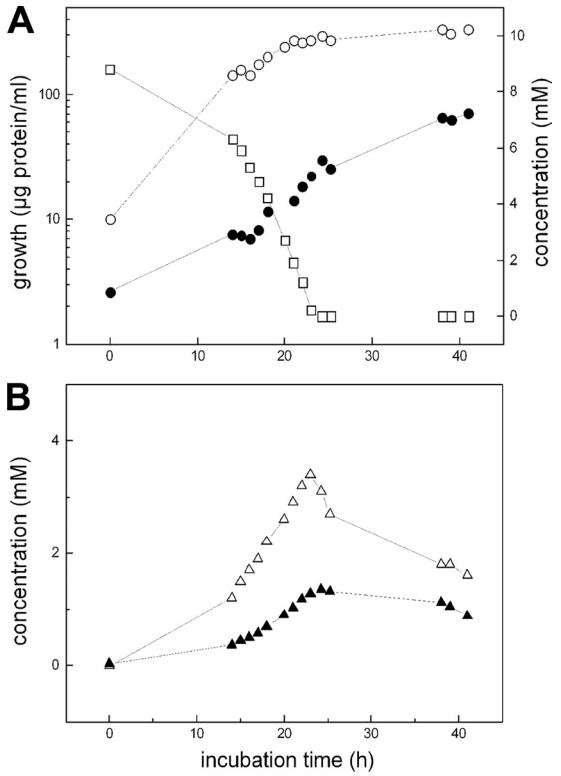

FIG. 3. Growth of *C. testosteroni* KF-1 with 3-C4-SPC under oxygen-limited conditions (see the text) (A), and transient excretion of two degradation intermediates (B) identified as 4-sulfoacetophenone (SAP) (Fig. 4) and 4-sulfophenol (SP) (see the text). Symbols: ○, total protein; □, 3-C4-SPC; ●, sulfate; △, SAP; ▲, SP.

The unknown metabolite present at lower concentration (Fig. 3B) had a UV spectrum identical to that of authentic 4-sulfophenol (λ_{max} 197, 231, and 271 nm) and cochromatographed with authentic 4-sulfophenol. This identified the metabolite as 4-sulfophenol (SP), which was an anticipated intermediate (Fig. 1) (28).

The major unknown metabolite eluted with a retention time (10.5 min) different from those of SP (3.5 min) and 3-C4-SPC (12.0 min). The UV spectrum (λ_{max}, 203, 258, and 286 nm) was shifted to longer wavelengths compared to the spectra of SP and SPC (28), but appeared similar to those observed for Δ2-enoyl 3-C4-SPC (3-C4-SPC-2H) or 4-sulfostyrene (cf. spectra in reference 28), thus suggesting a compound with an enhanced delocalization of the π electron system compared to

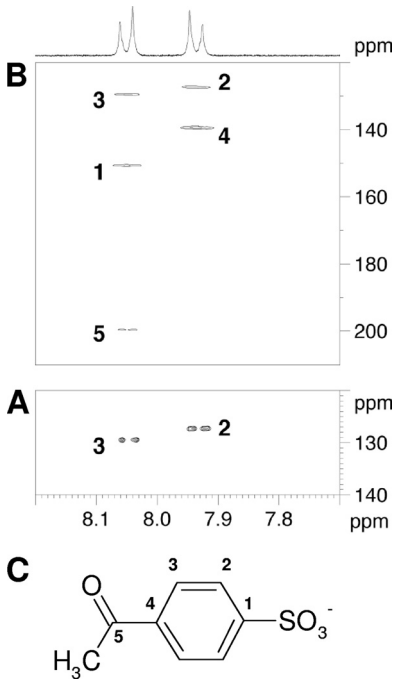

FIG. 4. Identification of 4-sulfoacetophenone by NMR. Shown are regions of interest of $^{1}H,^{13}C$-heteronuclear single quantum coherence (HSQC) (A) and HMBC (B) 2D NMR spectra recorded in CD_3OD solution (the regions with the resonances of the methyl group are not shown) with signal assignments (C) to the chemical structure of 4-sulfoacetophenone [4-acetylbenzene-sulfonate] isolated from the culture fluid.

SPC. The compound was purified by HPLC, and its chemical structure was evaluated by means of 1D and 2D correlated NMR spectra (Fig. 4A and B). From the chemical shifts determined and the observed $^{1}H,^{13}C$ heteronuclear multiple bond correlations (HMBCs), the structure of 4-sulfoacetophenone (4-acetylbenzenesulfonate) was postulated (Fig. 4C). The NMR spectra of commercially obtained, authentic 4-sulfoacetophenone corresponded exactly to the data obtained from the isolated metabolite, and also the UV spectrum and the HPLC retention time were identical (data not shown). Hence, the novel metabolite was 4-sulfoacetophenone (SAP).

C. testosteroni KF-1 grew exponentially with SP or SAP as the sole carbon and energy sources in fully aerated cultures (data not shown), and in both cases, quantitative growth and closed mass balances for carbon and sulfur (>95% released as sulfate) were observed. During growth under suboptimal aeration with SAP (data not shown), transient release of SP could be detected (up to 0.2 mM); no release of intermediates could be detected during growth with SP under these conditions (data not shown). Dense suspensions of washed cells were prepared from a 3-C4-SPC-grown culture, suboptimally aerated (2-ml scale, optical density [OD] $\approx$ 2, marginally stirred), and 3-C4-SPC was added (1 mM). The reactions were followed discontinuously by HPLC (data not shown). Both SAP and SP were excreted. In addition, a further unknown compound was detected at low levels (retention time, 2.9 min; λ_{max}, 204, 235, and 282 nm). This compound was identified by cochromatography with authentic material and by its characteristic UV spectrum as 4-sulfocatechol (SC), another anticipated intermediate (Fig. 1) (28).

Strain KF-1 grew well with 3-C4-SPC, SAP, and SP, but not with SC. SP-grown cells are known to express the degradative pathway for 3-C4-SPC, whereas succinate-grown cells do not (28). Because only SP was available as a bulk chemical, we routinely used this substrate as the carbon source to generate large quantities of cells of strain KF-1 induced for the degradative pathway of 3-C4-SPC.

Activities of oxygenases and an esterase in *C. testosteroni* KF-1. Suspensions of cells induced to utilize 3-C4-SPC were tested for their ability to oxidize potential intermediates in the degradative pathway (Table 1). The four compounds tested all caused reproducible oxygen uptake, but the specific rate with 3-C4-SPC (1.9 mkat [kg protein]$^{-1}$) was an order of magnitude higher than that with SAP, SP, or SC (Table 1). Samples from the oxygen electrode were examined by HPLC, which confirmed that these substrates were degraded. In parallel experiments under an atmosphere of N_2, no substrate disappearance was observed until air was added (data not shown).

The same four compounds were examined as substrates for unamended crude extracts from induced cells. In such experiments, only the addition of SC caused oxygen consumption (Table 1). Addition of 3-C4-SPC did not cause oxygen uptake under any condition tested (Table 1). SAP caused only NADPH-dependent oxygen uptake, while SP caused NADH- and NADPH-dependent oxygen uptake, with NADH allowing for higher rates (Table 1). We postulate that 3-C4-SPC required energy-dependent activation for further metabolism (see below), whereas SAP, SP, and SC were subject to oxygenation.

The putative NADPH-dependent SAP-oxygenase was found to be present in the soluble fraction of the cell extract (0.39 mkat [kg protein]$^{-1}$); the membrane fraction contained no activity. When this reaction was discontinuously followed in crude extract, the products acetate and SP were quantitatively recovered (data not shown). We presume that this represents the activities of two enzymes, a Baeyer-Villiger-type SAP-monooxygenase and an esterase. This hypothesis was sup-

TABLE 1. Oxygenase activities detected in whole cells and cell extract

Substrate(s)	Sp act (mkat [kg protein]$^{-1}$)	
	Whole cells	Cell extract
3-C4-SPC	1.9 ± 0.5	<0.01
3-C4-SPC + NADPH	NA[a]	<0.01
3-C4-SPC + NADH	NA	<0.01
SAP	0.20 ± 0.09	<0.01
SAP + NADPH	NA	0.16
SAP + NADH	NA	<0.01
SP	0.14 ± 0.05	<0.01
SP + NADPH	NA	0.18
SP + NADH	NA	0.38
SC	0.23 ± 0.08[b]	0.21[b]

[a] NA, not applicable.
[b] No yellow color development was observed.

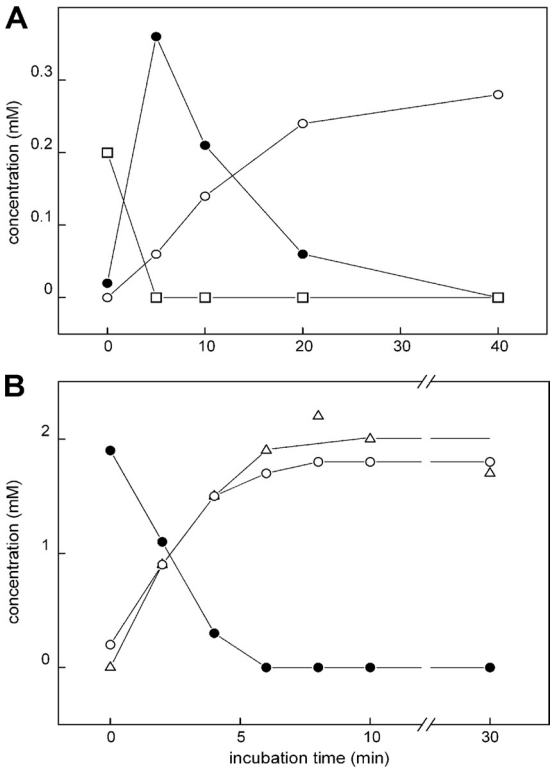

FIG. 5. NADPH-dependent transformation of SAP to SP with transient appearance of 4-sulfophenol acetate (A) and transformation of 4-sulfophenol acetate to SP and acetate (B). (A) A fraction of cell extract with SAP-oxygenase activity obtained from column-purification was used for the reaction; the initial SAP concentration was 0.3 mM. (B) Transformation of SPAc to SP and acetate was followed in cell extract. Symbols: □, SAP; ●, 4-sulfophenol acetate (SPAc); ○, SP; △, acetate.

ported when partially purified SAP-monooxygenase was incubated with SAP and NADPH. In such incubations, a transient intermediate could be detected along with acetate and SP formation (Fig. 5A). This intermediate was identified as 4-sulfophenol acetate (SPAc) (1-phenol-4-sulfonate-acetate), as it had a UV spectrum (λ_{max}, 197, 223, and 265 nm) identical to that of authentic SPAc and it cochromatographed (11.2 min) with authentic SPAc (data not shown). This compound, which was stable in the absence of crude extract, was hydrolyzed quantitatively by crude extract to acetate and SP (Fig. 5B); acetate (as acetic acid) was identified by cochromatography (gas chromatography [GC]) with authentic material. Finally, *C. testosteroni* KF-1 grew exponentially with SPAc when tested (not shown), and mass balance for carbon (5.2 g protein [mol C]$^{-1}$) and sulfur (>90% released as sulfate) was obtained in these growth experiments.

The NAD(P)H-dependent SP-oxygenase activity and also the SC-dioxygenase activity (Table 1) were found to be present in the soluble fraction of the crude extract (data not shown), but the activities were lost upon partial purification by anion-exchange chromatography under the conditions we used (data not shown).

DISCUSSION

It has been known for many years that SPCs and phenylcarboxylates are intermediates in the degradation of LAS and linear alkylbenzenes (LAB), respectively, but the degradation pathway of short-chain SPCs and phenylcarboxylates resisted all attempts at elucidation to date (1, 5, 31, 37). Our earlier work on the degradation of commercial LAS and individual SPCs in mixed and pure cultures, respectively, led us to anticipate that 3-C4-SPC should be quantitatively degraded and that the optical isomers should be turned over at different rates (Fig. 2) (6, 28, 30). Furthermore, we concluded that SP and SC are involved as metabolites (Fig. 6, reactions G and H) and should be transformed in agreement with the *ortho*-degradation pathway and desulfonation reactions established elsewhere (2, 10, 11) (Fig. 6, reactions I to M) but shown also to be active in SPC-degrading organisms (6, 28, 30).

So far, the missing part from the postulation of a reasonable degradation pathway for SPCs was the identification of reactions for the removal of the carboxylate side chain in SPCs. Here, we present data that strongly support the involvement of a Bayer-Villiger-type monooxygenase (BVMO) reaction in the metabolism of 3-C4-SPC by strain KF-1. The postulated degradation pathway is depicted in Fig. 6.

Suboptimal aeration of cultures (Fig. 3) (or of cell suspensions) of strain KF-1 affected the accumulation of a novel metabolite, which was unambiguously identified as SAP (Fig. 4; compound VI in Fig. 6). This metabolite provided the missing link for the elucidation of the pathway and allowed us to conclude that the C4 side chain of 3-C4-SPC was removed stepwise as C_2 moieties (reactions D and F in Fig. 6).

We were able to show that the second C_2 moiety to be removed was acetate (Fig. 5), which is a growth substrate for strain KF-1. The observed enzymatic hydrolysis of SPAc (Fig. 5; reaction F in Fig. 6), which was also unambiguously identified, to yield SP explains our earlier observations with regard to the presence of SP (and/or SC) in spent growth media (28, 30). We postulate that the ester SPAc is generated from SAP by an oxygenase, presumably a Baeyer-Villiger-type monooxygenase (BVMO) (Fig. 6, reaction E), in analogy to the known bacterial degradative pathway for 4-hydroxyacetophenone (HAP), a structural analogue of SAP. HAP is converted into 4-hydroxyphenyl acetate (HPAc) through insertion of an oxygen atom between the keto-carbon and the adjacent ring-carbon to form an ester, which is subsequently hydrolyzed to acetate and hydroquinone by an esterase (3, 13, 21, 24, 34).

From the results of the oxygen consumption experiments with crude extract (Table 1), in which 3-C4-SPC did not effect any oxygen consumption, it seems reasonable to assume that 3-C4-SPC needs to be activated before further oxygenation reactions could take place. Analogous to β-oxidation, we postulate formation of CoA esters (Fig. 6, reactions A). Further metabolism would be in analogy to β-oxidation: e.g., formation of a double bond (reaction B in Fig. 6). Metabolism of the different substrates (*R*)- and (*S*)-3-C-4-SPC and 3-C-4-SPC-2H would converge at the enoyl-CoA ester. Again analogous to β-oxidation, water would be added across the double bond (Fig. 6, reaction C). The subsequent release of acetyl-CoA to yield SAP would be a keto-acid lyase reaction (reaction D in Fig. 6). One precedent for such a reaction is 3-hydroxy-3-methylglutaryl-CoA lyase (EC 4.1.3.4) in a leucine degradation pathway; the enzyme cleaves 3-hydroxy-3-methylglutaryl-CoA into acetyl-CoA and the corresponding ketone, acetoacetate (e.g., see reference 8). Similarly, a modified

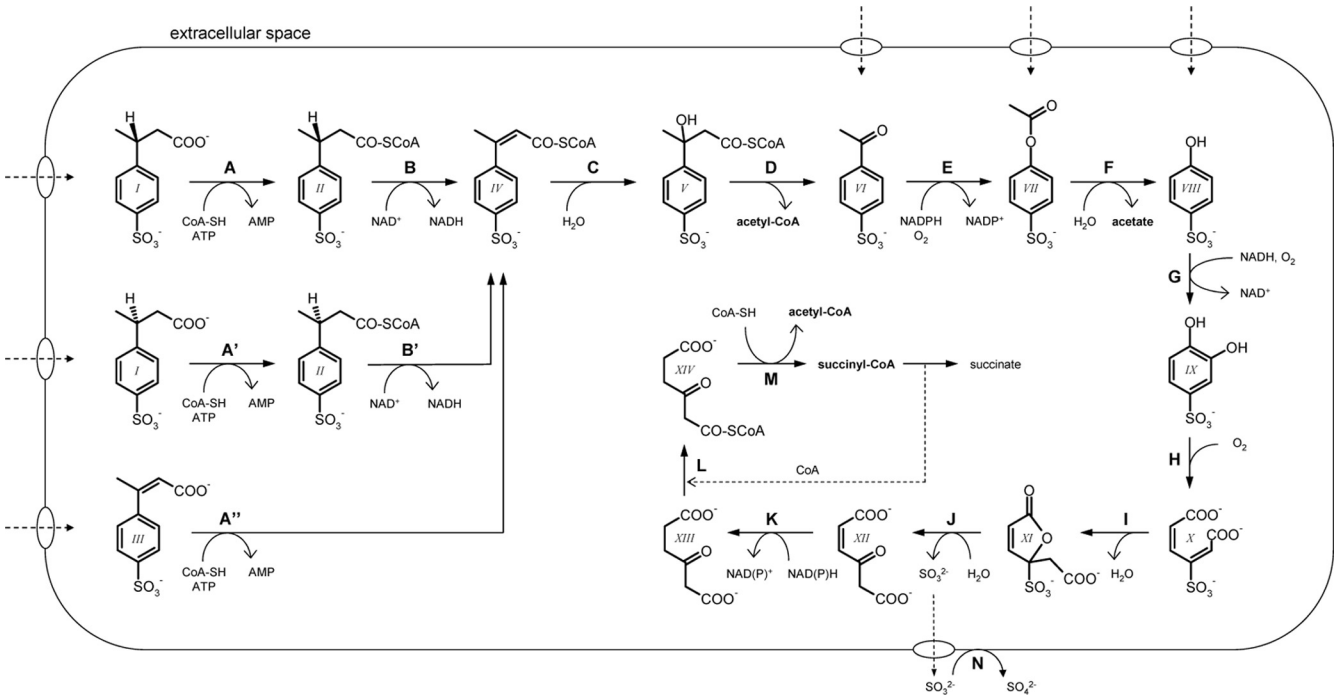

FIG. 6. Postulated degradation pathway for (*R*, *S*)-3-C4-SPC and 3-C4-SPC-2H in *C. testosteroni* KF-1. The transport systems inferred from the ability of strain KF-1 to utilize (*R*,*S*)-3-C4-SPC, 3-C4-SPC-2H, SAP, SPAc, and SP as growth substrates, are also indicated. Enzymes: A, acyl-CoA synthetase (or acyl-CoA transferase?); B, acyl-CoA dehydrogenase; C, enoyl-CoA hydratase; D, keto-acid-lyase; E, Baeyer-Villiger-type monooxygenase; F, esterase; G, 4-sulfophenol 2-monooxygenase; H, 4-sulfocatechol 1,2-dioxygenase; I, 3-sulfomuconate cycloisomerase; J, 4-sulfomuconolactone hydrolase (desulfonating); K, maleylacetate reductase; L, succinyl-CoA:3-ketoadipate CoA-transferase; M, 3-ketoadipyl-CoA thiolase; N, sulfite dehydrogenase. Compounds: I, (*R*,*S*)-3-C4-SPC [(*R*,*S*)-3-(4-sulfophenyl)butyrate]; II, (*R*,*S*)-3-C4-SPC-CoA; III, 3-C4-SPC-2H [3-(4-sulfophenyl)-Δ2-enoyl-butyrate; 3-(4-sulfophenyl)crotonate]; IV, 3-C4-SPC-2H-CoA; V, 3-C4-SPC-OH-CoA [3-hydroxy-3-(4-sulfophenyl)-butyryl-CoA]; VI, SAP (4-sulfoacetophenone); VII, SPAc (4-sulfophenol acetate); VIII, SP (4-sulfophenol); IX, SC (4-sulfocatechol); X, 3-sulfomuconate; XI, 4-sulfomuconolactone; XII, maleylacetate; XIII, 3-ketoadipate; and XIV, 3-ketoadipyl-CoA.

leucine pathway in *Vibrio* sp. involves 3-hydroxy-3-methylbutanoyl-CoA lyase (EC 4.1.3.-), which yields acetyl-CoA and the corresponding ketone, acetone (23). We therefore suggest that a 3-hydroxy-3-(4-sulfophenyl)butyryl-CoA lyase catalyzes reaction D.

Sulfonates must be transported into the cell (9), and transport systems corresponding to the sulfonate utilization range exhibited by *C. testosteroni* for the uptake of (*R*,*S*)-3-C4-SPC, 3-C4-SPC-2H, SAP, SPAc, and SP need to be present in this organism (Fig. 6). In addition, a sulfite exporter and a sulfite dehydrogenase can be anticipated (4) (Fig. 6).

LAS has been used for over 50 years (see the introduction), so ever since, SPCs have been produced and degraded in the environment, primarily in sewage treatment systems. Now, for the first time, we are able to supply a reasonable proposition for the complete degradative pathway of distinct SPCs (Fig. 6). It must be emphasized that some parts of the proposed pathway (Fig. 6) still need further experimental corroboration and thus must be considered as useful hypotheses for now. But it is also a limited hypothesis, since several important questions with regard to microbial metabolism of LAS congeners still remain unsolved. For example, whether *C. testosteroni* KF-1 metabolizes 3-C5-SPC (Fig. 1) along the 3-C4-SPC pathway (analogous to reactions A to C in Fig. 6), but with formation of 4-sulfopropiophenone instead of SAP, needs to be tested. It also remains unclear how the longer-chain SPCs, such as (*R*,*S*)-4-C6-SPC in *Delftia acidovorans* SPH-1 (28), are degraded when a "simple" α,β-unsaturation (reaction B in Fig. 6) would not eliminate the chirality of the compounds. Furthermore, it remains unclear how (and by which organisms) the many SPdCs are degraded, which are formed from centrally substituted LAS congeners (27). It is quite obvious that much more work is needed to completely uncover the metabolic pathways of all SP(d)C intermediates that are transiently formed during LAS degradation.

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REFERENCES

1. **Cain, R. B.** 1987. Biodegradation of anionic surfactants. Biochem. Soc. Trans. **15:**7–22.
2. **Contzen, M., S. Bürger, and A. Stolz.** 2001. Cloning of the genes for a 4-sulphocatechol-oxidizing protocatechuate 3,4-dioxygenase from *Hydrogenophaga intermedia* S1 and identification of the amino acid residues responsible for the ability to convert 4-sulphocatechol. Mol. Microbiol. **41:**199–205.
3. **Darby, J. M., D. G. Taylor, and D. J. Hopper.** 1987. Hydroquinone as the ring-fission substrate in the catabolism of 4-ethylphenol and 4-hydroxyacetophenone by *Pseudomonas putida* JD1. J. Gen. Microbiol. **133:**2137–2146.
4. **Denger, K., S. Weinitschke, T. H. Smits, D. Schleheck, and A. M. Cook.** 2008. Bacterial sulfite dehydrogenases in organotrophic metabolism: separation and identification in *Cupriavidus necator* H16 and in *Delftia acidovorans* SPH-1. Microbiology **154:**256–263.

5. **Dodgson, K. S., and F. F. White.** 1983. Some microbial enzymes involved in the biodegradation of sulphated surfactants. Top. Enzyme Ferment. Biotechnol. **7:**90–155.
6. **Dong, W., P. Eichhorn, S. Radajewski, D. Schleheck, K. Denger, T. P. Knepper, J. C. Murrell, and A. M. Cook.** 2004. *Parvibaculum lavamentivorans* converts linear alkylbenzenesulfonate (LAS) surfactant to sulfophenylcarboxylates, α,β-unsaturated sulfophenylcarboxylates and sulfophenyldicarboxylates, which are degraded in communities. J. Appl. Microbiol. **96:**630–640.
7. **Eichhorn, P., and T. P. Knepper.** 2002. α,β-Unsaturated sulfophenylcarboxylate intermediates detected during aerobic degradation of linear alkylbenzenesulfonate (LAS) surfactant: direct evidence for ω-oxygenation followed by β-oxidations by liquid chromatography-electrospray mass spectrometry. Environ. Toxicol. Chem. **21:**1–8.
8. **Forouhar, F., M. Hussain, R. Farid, J. Benach, M. Abashidze, W. C. Edstrom, S. M. Vorobiev, R. Xiao, T. B. Acton, Z. Fu, J. J. Kim, H. M. Miziorko, G. T. Montelione, and J. F. Hunt.** 2006. Crystal structures of two bacterial 3-hydroxy-3-methylglutaryl-CoA lyases suggest a common catalytic mechanism among a family of TIM barrel metalloenzymes cleaving carbon-carbon bonds. J. Biol. Chem. **281:**7533–7545.
9. **Graham, D. E., H. Xu, and R. H. White.** 2002. Identification of coenzyme M biosynthetic phosphosulfolactate synthase: a new family of sulfonate biosynthesizing enzymes. J. Biol. Chem. **277:**13421–13429.
10. **Halak, S., T. Basta, S. Burger, M. Contzen, and A. Stolz.** 2006. Characterization of the genes encoding the 3-carboxy-cis, cis-muconate-lactonizing enzymes from the 4-sulfocatechol degradative pathways of *Hydrogenophaga intermedia* S1 and *Agrobacterium radiobacter* S2. Microbiology **152:**3207–3216.
11. **Halak, S., T. Basta, S. Burger, M. Contzen, V. Wray, D. H. Pieper, and A. Stolz.** 2007. 4-Sulfomuconolactone hydrolases from *Hydrogenophaga intermedia* S1 and *Agrobacterium radiobacter* S2. J. Bacteriol. **189:**6998–7006.
12. **Hrsák, D., and A. Begonja.** 2000. Possible interactions within a methanotrophic-heterotrophic groundwater community able to transform linear alkylbenzenesulfonates. Appl. Environ. Microbiol. **66:**4433–4439.
13. **Kamerbeek, N. M., M. J. Moonen, J. G. Van Der Ven, W. J. Van Berkel, M. W. Fraaije, and D. B. Janssen.** 2001. 4-Hydroxyacetophenone monooxygenase from *Pseudomonas fluorescens* ACB. A novel flavoprotein catalyzing Baeyer-Villiger oxidation of aromatic compounds. Eur. J. Biochem. **268:**2547–2557.
14. **Kennedy, S. I. T., and C. A. Fewson.** 1968. Enzymes of the mandelate pathway in bacterium N.C.I.B. 8250. Biochem. J. **107:**497–506.
15. **Knepper, T. P., and J. L. Berna.** 2003. Surfactants: properties, production, and environmental aspects, p. 1–50. *In* T. P. Knepper, D. Barceló, and P. de Voogt (ed.), Analysis and fate of surfactants in the aquatic environment. Elsevier, Amsterdam, The Netherlands.
16. **Kölbener, P., U. Baumann, T. Leisinger, and A. M. Cook.** 1995. Non-degraded metabolites arising from the biodegradation of commercial linear alkylbenzenesulfonate (LAS) surfactants in a laboratory trickling filter. Environ. Toxicol. Chem. **14:**561–569.
17. **Laue, H., K. Denger, and A. M. Cook.** 1997. Taurine reduction in anaerobic respiration of *Bilophila wadsworthia* RZATAU. Appl. Environ. Microbiol. **63:**2016–2021.
18. **Laue, H., J. A. Field, and A. M. Cook.** 1996. Bacterial desulfonation of the ethanesulfonate metabolite of the chloroacetanilide herbicide metazachlor. Environ. Sci. Technol. **30:**1129–1132.
19. **Lunar, L., S. Rubio, and D. Perez-Bendito.** 2004. Differentiation and quantification of linear alkyl benzenesulfonate isomers by liquid chromatography-ion-trap mass spectrometry. J. Chromatogr. A **1031:**17–25.
20. **Matthijs, E., and H. De Henau.** 1987. Determination of LAS. Tenside Surfactants Detergents **24:**193–198.
21. **Moonen, M. J., N. M. Kamerbeek, A. H. Westphal, S. A. Boeren, D. B. Janssen, M. W. Fraaije, and W. J. van Berkel.** 2008. Elucidation of the 4-hydroxyacetophenone catabolic pathway in *Pseudomonas fluorescens* ACB. J. Bacteriol. **190:**5190–5198.
22. **Müller, T. A., and H.-P. E. Kohler.** 2004. Chirality of pollutants—effects on metabolism and fate. Appl. Microbiol. Biotechnol. **64:**300–316.
23. **Nemecek-Marshall, M., C. Wojciechowski, W. P. Wagner, and R. Fall.** 1999. Acetone formation in the *Vibrio* family: a new pathway for bacterial leucine catabolism. J. Bacteriol. **181:**7493–7499.
24. **Rehdorf, J., C. L. Zimmer, and U. T. Bornscheuer.** 2009. Cloning, expression, characterization, and biocatalytic investigation of the 4-hydroxyacetophenone monooxygenase from *Pseudomonas putida* JD1. Appl. Environ. Microbiol. **75:**3106–3114.
25. **Sawyer, C. N., and D. W. Ryckman.** 1957. Anionic synthetic detergents and water supply problems. J. Am. Water Works Assoc. **49:**480–490.
26. **Schleheck, D., and A. M. Cook.** 2003. Saccharin as a sole source of carbon and energy for *Sphingomonas xenophaga* SKN. Arch. Microbiol. **179:**191–196.
27. **Schleheck, D., T. Knepper, P. Eichhorn, and A. M. Cook.** 2007. *Parvibaculum lavamentivorans* DS-1^T degrades centrally-substituted congeners of commercial linear alkylbenzenesulfonate (LAS) to sulfophenyl carboxylates and sulfophenyl dicarboxylates. Appl. Environ. Microbiol. **73:**4725–4732.
28. **Schleheck, D., T. P. Knepper, K. Fischer, and A. M. Cook.** 2004. Mineralization of individual congeners of linear alkylbenzenesulfonate (LAS) by defined pairs of heterotrophic bacteria. Appl. Environ. Microbiol. **70:**4053–4063.
29. **Schleheck, D., B. Tindall, R. Rosselló-Mora, and A. M. Cook.** 2004. *Parvibaculum lavamentivorans* gen. nov., spec. nov., a novel heterotroph that initiates catabolism of linear alkylbenzenesulfonate. Int. J. Syst. Evol. Microbiol. **54:**1489–1497.
30. **Schulz, S., W. Dong, U. Groth, and A. M. Cook.** 2000. Enantiomeric degradation of 2-(4-sulfophenyl)butyrate via 4-sulfocatechol in *Delftia acidovorans* SPB1. Appl. Environ. Microbiol. **66:**1905–1910.
31. **Simoni, S., S. Klinke, C. Zipper, W. Angst, and H.-P. E. Kohler.** 1996. Enantioselective metabolism of chiral 3-phenylbutyric acid, an intermediate of linear alkylbenzene degradation, by *Rhodococcus rhodochrous* PB1. Appl. Environ. Microbiol. **62:**749–755.
32. **Sörbo, B.** 1987. Sulfate: turbidimetric and nephelometric methods. Methods Enzymol. **143:**3–6.
33. **Swisher, R. D.** 1970. Surfactant biodegradation, 1st ed. Marcel Dekker, New York, NY.
34. **Tanner, A., and D. J. Hopper.** 2000. Conversion of 4-hydroxyacetophenone into 4-phenyl acetate by a flavin adenine dinucleotide-containing Baeyer-Villiger-type monooxygenase. J. Bacteriol. **182:**6565–6569.
35. **Thurnheer, T., T. Köhler, A. M. Cook, and T. Leisinger.** 1986. Orthanilic acid and analogues as carbon sources for bacteria: growth physiology and enzymic desulphonation. J. Gen. Microbiol. **132:**1215–1220.
36. **van Ginkel, C. G.** 1996. Complete degradation of xenobiotic surfactants by consortia of aerobic organisms. Biodegradation **7:**151–164.
37. **White, G. F., and N. J. Russell.** 1994. Biodegradation of anionic surfactants and related molecules, p. 143–177. *In* C. Ratledge (ed.), Biochemistry of microbial degradation. Kluwer Academic Publishers, Dordrecht, the Netherlands.