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Two Enzymes of a Complete Degradation Pathway for Linear Alkylbenzenesulfonate (LAS) Surfactants: 4-Sulfoacetophenone Baeyer-Villiger Monooxygenase and 4-Sulfophenylacetate Esterase in *Comamonas testosteroni* KF-1

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Complete biodegradation of the surfactant linear alkylbenzenesulfonate (LAS) is accomplished by complex bacterial communities in two steps. First, all LAS congeners are degraded into about 50 sulfophenylcarboxylates (SPC), one of which is 3-(4-sulfophenyl)butyrate (3-C_4-SPC). Second, these SPCs are mineralized. 3-C_4-SPC is mineralized by *Comamonas testosteroni* KF-1 in a process involving 4-sulfoacetophenone (SAP) as a metabolite and an unknown inducible Baeyer-Villiger monooxygenase (BVMO) to yield 4-sulfophenyl acetate (SPAc) from SAP (SAPMO enzyme); hydrolysis of SPAc to 4-sulfophenol and acetate is catalyzed by an unknown inducible esterase (SPAc esterase). Transcriptional analysis showed that one of four candidate genes for BVMOs in the genome of strain KF-1, as well as an SPAc esterase candidate gene directly upstream, was inducibly transcribed during growth with 3-C_4-SPC. The same genes were identified by enzyme purification and peptide fingerprinting-mass spectrometry when SAPMO was enriched and SPAc esterase purified to homogeneity by protein chromatography. Heterologously overproduced pure SAPMO converted SAP to SPAc and was active with phenylacetone and 4-hydroxyacetophenone but not with cyclohexanone and progesterone. SAPMO showed the highest sequence homology to the archetypal phenylacetone BVMO (57%), followed by steroid BVMO (55%) and 4-hydroxyacetophenone BVMO (30%). Finally, the two pure enzymes added sequentially, SAPMO with NADPH and SAP, and then SPAc esterase, catalyzed the conversion of SAP via SPAc to 4-sulfophenol and acetate in a 1:1:1:1 molar ratio. Hence, the first two enzymes of a complete LAS degradation pathway were identified, giving evidence for the recruitment of members of the very versatile type I BVMO and carboxylester hydrolase enzyme families for the utilization of a xenobiotic compound by bacteria.

Linear alkylbenzenesulfonate (LAS) is the major synthetic laundry surfactant in worldwide use, with an annual production of approximately 3 million tons per year (22). The commercial preparation of LAS is nominally constituted of a mixture of 20 congeners of secondary 4-sulfophenylalkanes (secondary C_{10}- to C_{13}-LAS), 18 of which are chiral (22). This complex mixture of congeners and enantiomers is completely degraded by complex heterotrophic aerobic bacterial communities in two steps (Fig. 1) (11, 40, 52). In the first step, bacteria utilize the alkyl chains of LAS for growth, by acquisition of acetyl coenzyme A (acetyl-CoA) through beta-oxidation of the alkyl moiety, and release a complex mixture of approximately 50 short-chain 4-sulfophenylcarboxylates (SPCs; C_4 to C_9 chain lengths) and related SPC-like intermediates, as observed with our model organism for primary LAS degradation, *Parvibaculum lavamentivorans* DS-1^T (37, 38, 41, 43). For an example relevant in this paper, the primary degradation of a C_{10}-LAS congener, (*R*,*S*)-4-sulfophenyldecane (2-C_{10}-LAS), by *P. lavamentivorans* DS-1^T yields (*R*,*S*)-3-(4-sulfophenyl)butyrate (3-C_4-SPC) as a major product (see Fig. 1 for the structures).

In the second step, the SPCs (and the other products) are completely degraded by other heterotrophic bacteria to cell material, CO_2, water, and sulfate (40, 42, 45). For this ultimate degradation step, our work with pure cultures of SPC-utilizing bacteria has shown that many different organisms must be active, because all known SPC-degrading representatives have a narrow substrate spectrum of only 3 to 4 individual SPC-like compounds (40, 45).

Any detailed information on the LAS and SPC degradation pathways, particularly the enzymes and genes involved, is still absent. In this study, we used the SPC-degrading bacterium *Comamonas testosteroni* KF-1 (40) and its available genome sequence (Genomes Online Database [GOLD] ID Gi01330), the proposed 3-C_4-SPC degradation pathway (see below), and the single SPC available as a substrate for strain KF-1 (chemically synthesized 3-C_4-SPC [42]) as our model system in the attempt to reveal the first enzymes and genes of a complete degradation pathway for LAS.

Our recent work (42) strongly suggested that an NADPH-dependent Baeyer-Villiger monooxygenase reaction is employed in the degradation pathway for 3-C_4-SPC in *C. testosteroni* KF-1 (Fig. 1). Baeyer-Villiger monooxygenases (BVMOs) are a specific type of oxidoreductase (EC 1.14.13.x), found in many different organisms, for the catalysis of a variety of oxidation reactions, including Baeyer-Villiger oxidations (1) in which aliphatic, cyclic, and/or aromatic ketones are converted to esters or lactones by the insertion of an oxygen atom into the higher substituted carbon-carbon bond of the carbonylic substrate (e.g., see reference 19). In the 3-C_4-SPC pathway in *C. testosteroni* KF-1, the identified carbon-

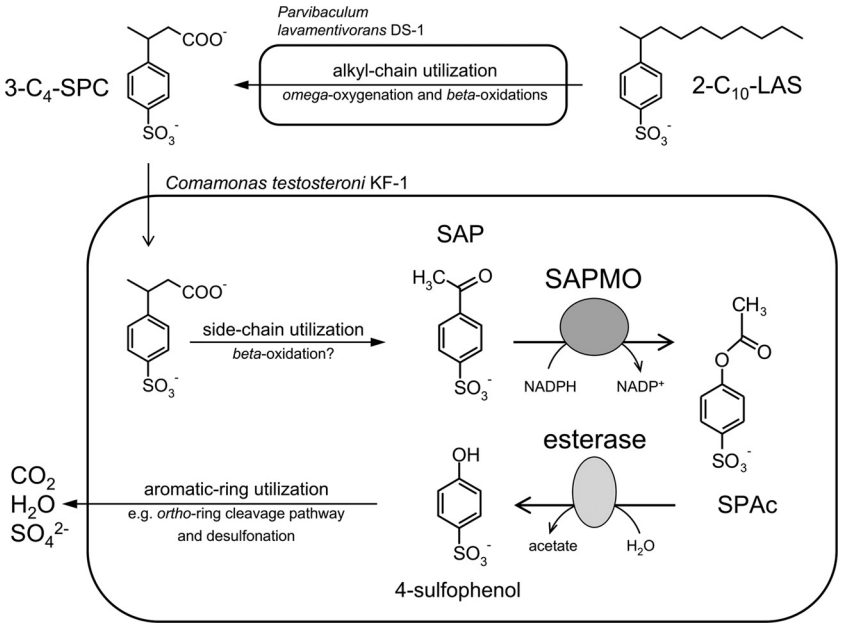


FIG 1 Schematic representation of the complete degradation of LAS surfactant by pairs of heterotrophic bacteria and illustration of the two enzyme reactions that are the topic of this study, involving a 4-sulfoacetophenone (SAP) Baeyer-Villiger-type monooxygenase (SAPMO) and a 4-sulfophenyl acetate (SPAc) esterase (SPAc esterase) in *Comamonas testosteroni* KF-1. LAS [here, the 2-(4-sulfophenyl)decane (2-C_{10}-LAS) congener is shown] is degraded in a first step to 4-sulfophenylcarboxylates (SPCs) [here, to 3-(4-sulfophenyl)butyrate (3-C_4-SPC)] by, e.g., *Parvibaculum lavamentivorans* DS-1^T (39, 41). In a second step, the SPCs are mineralized by other bacteria, represented here by *Comamonas testosteroni* KF-1, which is able to utilize 3-C_4-SPC for growth (40). None of the enzymes or genes for LAS and SPC degradation has been identified thus far.

ylic metabolite 4-sulfoacetophenone (SAP; 4-acetylbenzenesulfonate) (Fig. 1) was indicated to be converted by an unknown, strictly NADPH-dependent SAP BVMO enzyme (SAPMO) to 4-sulfophenyl acetate (SPAc) (Fig. 1) (42). The ester, which is stable, was shown to be cleaved by an unknown esterase to yield 4-sulfophenol (4-hydroxybenzenesulfonate) and acetate (Fig. 1) (42). The precedents for this reaction sequence in strain KF-1 (Fig. 1) can be found in *Pseudomonas* strains that convert a structural analog of SAP, carbonylic 4-hydroxyacetophenone (HAP), to 4-hydroxyphenyl acetate (HPAc) using an NADPH-dependent HAP BVMO enzyme (HAPMO), which is subsequently hydrolyzed to hydroquinone and acetate by an esterase (HPAc esterase) (20, 28, 33, 34). HAPMO belongs to a large family of so-called type I BVMOs within the family of flavoprotein monooxygenases; these enzymes are NADPH dependent, contain flavin adenine dinucleotide (FAD), and share a highly conserved "BVMO fingerprint" sequence motif (12). Type II BVMOs use NADH and flavin mononucleotide (FMN) and contain no BVMO fingerprint motif (25).

The BVMO-esterase reaction sequence from SAP via SPAc to 4-sulfophenol and acetate in *C. testosteroni* KF-1 (Fig. 1) allowed for the first proposition of a reasonable SPC degradation pathway in bacteria (42). Briefly, the upper part of the 3-C_4-SPC pathway leading to SAP is thought to involve the utilization of a first C_2 unit from the C_4 side chain of 3-C_4-SPC, most likely by a reaction sequence in analogy to beta-oxidation and, thus, an abstraction of acetyl-CoA (see schematic representation in Fig. 1) (for details and structures, see reference 42). The lower 3-C_4-SPC pathway, i.e., after the proposed BVMO-esterase reaction sequence and from 4-sulfophenol further via aromatic ring cleavage to central metabolites, is thought to proceed via 4-sulfophenol 2-monooxygenation to 4-sulfocatechol, followed by 4-sulfocatechol *ortho*-ring cleavage (9, 15, 16) (see schematic representation in Fig. 1) (for details and structures, see reference 42).

In the present study, we focused on the two readily accessible enzyme reactions in the 3-C_4-SPC degradation pathway in *C. testosteroni* KF-1, catalyzed by predicted SAPMO and SPAc esterase, and report that the genome sequence of *C. testosteroni* KF-1, kindly made available by the Joint Genome Institute of the U.S. Department of Energy (DOE-JGI), contains four valid candidate genes for BVMOs and that one of these genes encodes SAPMO. The SPAc esterase was also identified.

MATERIALS AND METHODS

Growth conditions. *C. testosteroni* KF-1 (DSM 14576) was isolated in our laboratory (40). A phosphate-buffered mineral salts medium (49) supplemented with 3-(4-sulfophenyl)butyrate (6 mM) or succinate (15 mM) as the sole carbon source was used. Cultures in the 3-ml scale were incubated in glass tubes (Corning) on a roller and cultures in the 0.1- to 2-liter scale in Erlenmeyer flasks on a shaker at 30°C in the dark. Cultures were inoculated (1%) with cultures pregrown with the same substrate.

Chemicals. Standard chemicals were obtained from Sigma-Aldrich, Fluka, or Merck. 4-Sulfoacetophenone (4-acetylbenzenesulfonate) was purchased from ABCR (Karlsruhe, Germany), 4-sulfophenol acetate (1-phenol-4-sulfonate-acetate) from Synchem (Felsberg-Altenburg, Germany), and biochemicals (NADH, NADPH, NAD^+, and $NADP^+$) from Biomol (Hamburg, Germany). Racemic 3-(4-sulfophenyl)butyrate was synthesized as described previously (40).

RNA preparation and RT-PCR. RNA preparation and reverse transcription (RT)-PCR were performed as described previously (35), with the following modifications. After cells were grown in the appropriate selective medium (5 ml) and harvested in the mid-exponential growth phase (optical density at 580 nm [OD_{580}] $\approx$ 0.3), the cell pellets were stored at −20°C in RNAlater RNA stabilization solution (Ambion, Ap-

plied Biosystems). Total RNA was prepared using the E.Z.N.A. bacterial RNA kit (Omega Bio-Tek) following the manufacturer's instructions; the RNA preparation obtained (40 μl) was treated with RNase-free DNase (2 U for 30 min at 37°C) (Fermentas). For cDNA synthesis, Moloney murine leukemia virus (MMLV) reverse transcriptase (Fermentas) was used following the manufacturer's instructions; the reaction mixtures contained 0.2 μg total RNA and 20 pmol sequence-specific primer. The primer sequences used are provided in Table S1 in the supplemental material and were purchased from Microsynth (Balgach, Switzerland). PCRs (20-μl scale) were done using *Taq* DNA polymerase (Fermentas) and the manufacturer's standard reaction mixture (including 2.5 mM $MgCl_2$). As the template, cDNA from RT reactions (2 μl of RT reaction mixture), genomic DNA (4 ng DNA) for PCR-positive controls, or non-reverse-transcribed total RNA (2 μl) for confirmation of the absence of DNA impurities in the RNA preparations was used.

Preparation of cell extracts and purification of native enzymes. *C. testosteroni*. KF-1 cells were harvested in the exponential growth phase by centrifugation (15,000 × *g* for 20 min at 4°C) and stored frozen (−20°C). Cells were resuspended in 50 mM Tris-H_2SO_4 buffer (pH 8.0 or pH 9.0) containing 0.03 mg ml^{-1} DNase I (Sigma) and 2 mM $MgCl_2$ and disrupted by three to four passages through a French pressure cell (140 MPa at 4°C) (Newport Scientific, Inc./AMINCO, Silver Spring, MD). Whole cells and debris were removed by centrifugation (15,000 × *g* for 20 min at 4°C) to obtain crude extract, and membranes were removed by ultracentrifugation (60,000 × *g* for 30 min at 4°C) to obtain the soluble fraction; the membrane pellets were resuspended in 50 mM Tris-H_2SO_4 buffer (pH 8.0 or pH 9.0) to obtain the membrane fraction.

For purification of native SPAc esterase, the salinity of the soluble fraction (in 50 mM Tris-H_2SO_4 buffer, pH 9.0) was increased to 1.7 M ammonium sulfate through dropwise addition of ammonium sulfate solution (3.4 M) while stirring on ice. The precipitated proteins were collected (15,000 × *g* for 20 min at 4°C), and the supernatant was passed over a PD10 column (Pharmacia) equilibrated with 50 mM Tris-H_2SO_4 buffer, pH 9.0, for desalting. The protein solution was then subjected to anion-exchange chromatography on a Mono Q HR (high-resolution) 10/10 column (Pharmacia) equilibrated with 50 mM Tris-H_2SO_4 buffer, pH 9.0, at a flow rate of 1 ml min^{-1}. Bound proteins were eluted by using a linear Na_2SO_4 gradient (to 0.2 M in 45 min and to 0.5 M in 10 min), and fractions (3 ml) were collected; the esterase activity eluted at about 80 mM Na_2SO_4 in two fractions. Active fractions were pooled, their salinity was increased to 1.7 M ammonium sulfate (see above), and they were subjected to hydrophobic interaction chromatography on a phenyl-Superose HR 10/10 column (Pharmacia) equilibrated with 1.7 M ammonium sulfate in 50 mM Tris-H_2SO_4 buffer, pH 8.5; the esterase activity eluted at about 150 mM Na_2SO_4 in two fractions, which were pooled and concentrated using a Vivaspin (10-kDa cutoff; Sartorius, Goettingen, Germany). Finally, gel filtration on a Superose 12 (HR 10/30; Pharmacia) was performed in 50 mM Tris-H_2SO_4 buffer, pH 9.0, including 150 mM sodium sulfate, at a flow rate of 0.4 ml min^{-1}. Standard high-molecular-weight proteins (aprotinin, RNase A, carbonic anhydrase, ovalbumin, conalbumin, and aldolase) were used to calibrate the column.

For separation of native SAPMO, the resuspension buffer (50 mM Tris-H_2SO_4, pH 8.0) was supplemented with FAD and $NADP^+$, each at 50 μM concentration, and 10% (vol/vol) glycerol. Anion-exchange chromatography on a Mono Q column and gel filtration were performed as described above, using 50 mM Tris-H_2SO_4 buffer, pH 8.0, supplemented by FAD and $NADP^+$ (100 μM each).

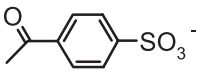
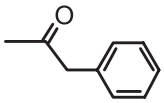
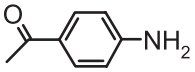
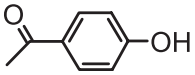
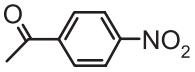
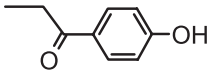
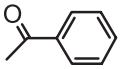
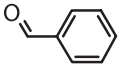
Heterologous expression of the *Comamonas testosteroni* SAPMO gene in *Escherichia coli* and purification of the recombinant protein. Chromosomal DNA was isolated by phenol-chloroform extraction, and the gene (locus tag CtesDRAFT_PD5437) amplified by PCR using Phusion HF DNA polymerase (Finnzymes) and a primer pair that is given in the supplemental material (see Table S1); the PCR conditions were 30 cycles of 20 s at 98°C for denaturation, 20 s at 58°C for annealing, and 90 s at 72°C for elongation. The PCR product was separated by agarose gel electrophoresis, excised, and purified using the QIAquick gel extraction kit (Qiagen), followed by ligation into an N-terminally His_6-tagged expression vector using the Champion pET100 directional TOPO expression kit (Invitrogen). OneShot TOP10 *E. coli* cells (Invitrogen) were transformed with the construct and the correct integration of the insert confirmed by sequencing (GATC-Biotech, Konstanz, Germany). For expression, BL21 Star (DE3) OneShot *E. coli* cells (Invitrogen) were transformed with the construct and grown at 37°C in LB medium containing 100 mg/liter ampicillin. At an OD_{580} of ≈0.6, the culture was induced by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside. The cells were grown for an additional 5 h at 20°C, harvested by centrifugation (15,000 × *g* for 20 min at 4°C), and stored frozen (−20°C). Cells were resuspended in buffer A (25 mM Tris-HCl, pH 8.0, 50 μM FAD, 50 μM $NADP^+$, 10% [vol/vol] glycerol) containing 0.03 mg ml^{-1} DNase I (Sigma) and disrupted by four passages through a precooled French pressure cell (140 MPa; Newport Scientific, Inc./AMINCO, Silver Spring, MD). Cell debris was removed by centrifugation (15,000 × *g* for 10 min at 4°C), and membranes by ultracentrifugation (60,000 × *g* for 1 h at 4°C). The soluble fraction was loaded on a Ni^+-chelating agarose affinity column (1-ml column volume; Macherey-Nagel, Germany) preequilibrated with buffer A (see above), followed by a washing step using 20 mM imidazole in buffer A. The His-tagged protein was eluted using 200 mM imidazole in buffer A, concentrated using a Vivaspin (50-kDa cutoff; Sartorius, Germany), and stored at −20°C after the addition of glycerol to a 30% (vol/vol) final concentration.

Enzyme assays. The activities of native and recombinant SAPMO were followed routinely as the decrease of absorption of the cosubstrate NADPH at 365 nm ($\varepsilon = 3.5 \times 10^3\ M^{-1}\ cm^{-1}$) (2) to avoid interference with substrates 4-hydroxyacetophenone, 4-aminoacetophenone, and 4-hydroxypropiophenone at 340 nm or, occasionally, as substrate disappearance by reversed-phase high-pressure liquid chromatography (HPLC) with UV detection (HPLC-UV; see below) or substrate-dependent oxygen consumption with a Clark-type oxygen electrode (42). The standard reaction mixture (1 ml) contained 50 mM Tris-HCl (pH 8.0), 0.5 mM NADPH, 0.5 mM SAP, and SAPMO as present in cell extracts or in partially purified preparations (2 to 50 μg total protein) or in the form of the purified recombinant enzyme (13 μg total protein). The K_m for NADPH was determined when its concentration was varied (from 0.005 to 0.3 mM) while keeping the SAP concentration constant (0.5 mM). The K_ms for SAP and other carbonylic substrates (Table 1) were determined when their concentrations were varied (SAP, 0.01 to 1 mM; phenylacetone [PA], 0.01 to 0.75 mM; 4-aminoacetophenone [AAP], 0.01 to 4 mM; 4-nitroacetophenone [NAP], 0.01 to 1 mM; 4-hydroxyacetophenone [HAP], 0.01 to 1 mM; 4-hydroxypropiophenone [HHP], 0.05 to 5 mM; acetophenone [AP], 0.5 to 20 mM; and benzaldehyde [BA], 0.05 to 5 mM) while keeping the NADPH concentration constant (0.5 mM). All substrate stocks were dissolved in ethanol at concentrations such that the final ethanol concentrations in the reaction mixtures did not exceed 2% (vol/vol). The activities were plotted using hyperbolic fit in Origin (Microcal Software, Inc.). The pH optimum of SAPMO was determined when the pH of the reaction buffer was varied from pH 5.2 to 10.5 (8). The thermostability of SAPMO was determined as described for phenylacetone BVMO (PAMO) (32), with the enzyme incubated at different temperatures (22 to 47°C) for 1 h, followed by the determination of activity under routine conditions (see above).

SPAc esterase was routinely spectrophotometrically measured as the increase of absorption of the reaction product 4-sulfophenol at 280 nm ($\varepsilon = 0.9\ mM^{-1}\ cm^{-1}$) or, occasionally, as substrate disappearance and product formation by HPLC, as described previously (42).

Analytical methods. SAP and other aromatic ketones tested (Table 1) and SPAc, NADPH, and $NADP^+$ were analyzed by reversed-phase HPLC with UV detection (HPLC-UV) using a Nucleosil C_{18} column (125 by 3 mm with a particle size of 5 μm; Macherey-Nagel, Germany) and a gradient system (mobile phase A, 20 mM potassium phosphate buffer, pH 2.2, and eluent B, 100% methanol, with a flow rate of 0.5 ml/min); the

TABLE 1 Substrate specificity and kinetic parameters determined for recombinant SAPMO from *Comamonas testosteroni* KF-1

Substrate[a]	Structure	Mean V_{max} ± SD (μmol min^{-1} mg^{-1})[b]	Mean K_m ± SD (μM)[b]	k_{cat} (s^{-1})	k_{cat}/K_m (10^3 s^{-1} M^{-1})
4-Sulfoacetophenone		2.8 ± 0.8	61.7 ± 11.5	2.9	46.6
Phenylacetone		2.7 ± 0.8	52.0 ± 8.0	2.8	53.7
4-Aminoacetophenone		1.7 ± 0.5	168.5 ± 23.7	1.8	10.5
4-Hydroxyacetophenone		1.1 ± 0.3	35.9 ± 9.6	1.1	8.2
4-Nitroacetophenone		1.1 ± 0.3	173.0 ± 45.2	1.1	30.4
4-Hydroxypropiophenone		0.4 ± 0.1	134.5 ± 32.9	0.4	2.5
Acetophenone		0.4 ± 0.1	558.8 ± 90.6	0.4	0.7
Benzaldehyde		0.4 ± 0.1	240.8 ± 90.8	0.4	1.7

[a] Substrate disappearance during the reactions was confirmed by HPLC-UV (data not shown).
[b] Determined as disappearance of cosubstrate NADPH (0.5 mM) in Tris-HCl buffer (pH 8.0, 50 mM) at 25°C.

gradient program was 100% A for 3 min to 35% B in 3 min to 75% B in 11 min, 75% B for 6 min to 100% A in 1 min, followed by reequilibration for 10 min. The retention times of the analytes were as follows: SAP, 8.1 min; SPAc, 8.4 min; 4-sulfophenol, 2.4 min; PA, 16.1 min; AAP, 10.2 min; HAP, 13.7 min; NAP, 14.8 min; HPP, 15.7 min; AP, 16.3 min; BA, 15.2 min; NADPH, 2.4 min; and $NADP^+$, 6.7 min. Acetate was determined by gas chromatography (24). Soluble protein was assayed by protein-dye binding (6). Denatured proteins were separated by 16% or 13% SDS-PAGE gels and stained with Coomassie brilliant blue R250 (23). Stained protein bands were cut from the gel and subjected to peptide fingerprinting-mass spectrometry (PF-MS) at the Proteomics Facility of the University of Konstanz (www.proteomics-facility.uni-konstanz.de) to identify the corresponding genes; the MASCOT engine (Matrix Science, London, United Kingdom) was used to search against a local amino acid sequence database of all annotated genes (DOE-JGI Integrated Microbial Genomes [IMG], 16 August 2011 version) and a local database of the translated (6 frames) nucleotide sequence of the genome of *Comamonas testosteroni* KF-1.

RESULTS

BVMO candidate genes in the genome of *C. testosteroni* KF-1. SAPMO is strictly NADPH dependent (42), and four valid candidate genes for NADPH-dependent (type I) BVMOs (Fig. 2A) were found in the unclosed draft genome sequence of strain KF-1 (6.026 Mbp, 5,492 protein-coding genes; unpublished data) when prototype NADPH-dependent (type I) Baeyer-Villiger monooxygenase sequences (e.g., of HAPMO) were used for the search (IMG BLASTp); the candidates share up to 57% amino acid sequence identity with representatives of the type I Baeyer-Villiger monooxygenases (see Discussion; for sequence alignment, see Fig. S1 in the supplemental material). Three of the four predicted BVMO genes (BVMO1 to BVMO3 genes) are located next to a candidate gene for a corresponding esterase (alpha/beta-fold hydrolase), either directly downstream on the same strand (*est1* and *est2*) or directly upstream in divergent orientation (*est3*) (Fig. 2A). Notably, the BVMO2 and BVMO3 gene candidates are each clustered with predicted genes for acyl-CoA metabolism, and the BVMO3 candidate gene cluster appears to be framed by two Tn*3* family transposase genes (Fig. 2A) that share >99% nucleotide sequence identity (see Discussion). Finally, the BVMO1 gene candidate is located next to the protocatechuate *meta*-ring cleavage pathway operon (Pmd operon) identified in a different *C. testosteroni* strain (31), and the BVMO4 gene candidate is clustered with putative genes for arsenic resistance (Fig. 2A).

Transcriptional analysis of BVMO gene candidates. SAPMO is inducibly expressed (42), and reverse transcription-PCR (RT-PCR) was used to test whether any of the attributed candidate BVMO genes is inducibly transcribed during growth with 3-C_4-SPC. The results (Fig. 2B) suggested that the candidate BVMO3 gene was strongly induced during growth with 3-C_4-SPC but not during growth with succinate, whereas no signals for the candidate BVMO1 and BVMO2 genes were detectable under any growth conditions tested; the BVMO4 gene candidate exhibited negligible transcription during both growth conditions tested. Furthermore, when tested by RT-PCR, a strong induction of esterase gene candidate *est3* was indicated (Fig. 2A) (data not shown) during growth with 3-C_4-SPC but not during growth with succinate. Hence, we had the first experimental support that the candidate BVMO3 and *est3* genes could encode SAPMO and SPAc esterase, respectively, in *C. testosteroni* KF-1.

Partial purification, some characteristics, and identification of SAPMO. The SAPMO activity in cell extracts of 3-C_4-SPC-

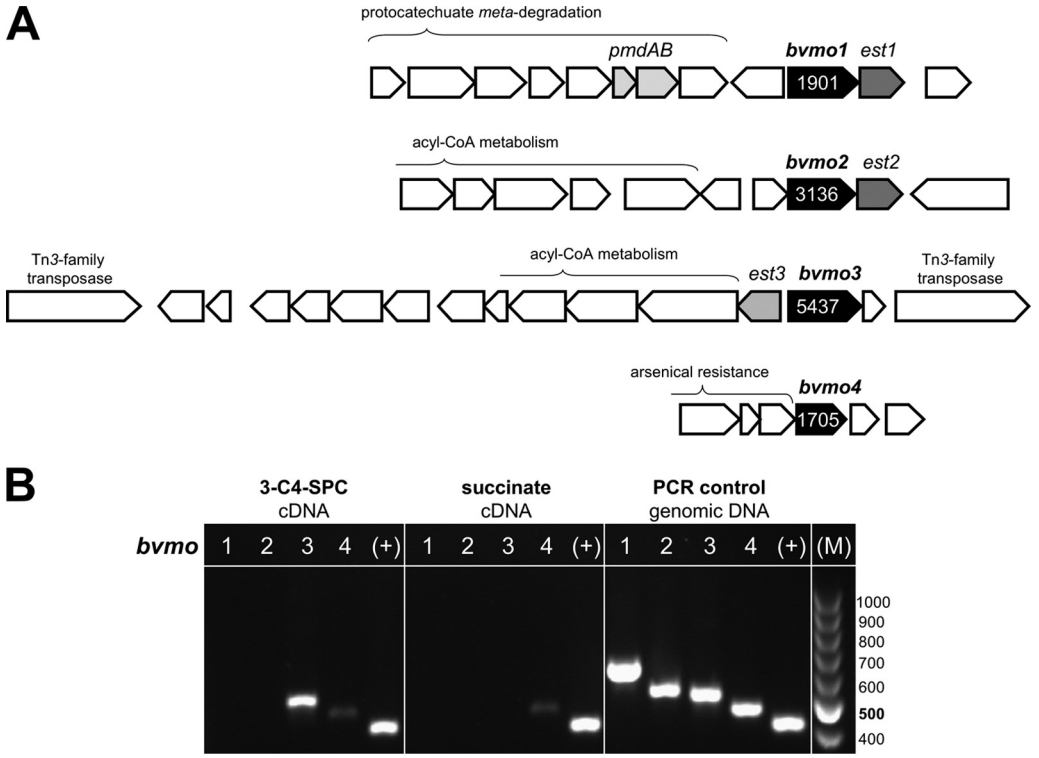


FIG 2 Illustration of clusters of genes in the *C. testosteroni* KF-1 genome that contain BVMO candidate genes (A), and analysis by reverse transcription-PCR of inducible transcription of the BVMO candidate genes in cells grown with 3-C_4-SPC in comparison to their transcription in succinate-grown cells (B). (A) The four BVMO gene candidates are indicated, and their locus tag numbers, not including the prefix CtesDRAFT_PD, are shown. Colocalized esterase gene candidates (*est1* to *est3*), as well as colocated genes for *meta*-ring cleavage dioxygenase (*pmdAB*) and degradation of protocatechuate, for acyl-CoA metabolism, for arsenic resistance, and for transposases of insertion (IS) elements (see the text), are also shown. (B) Agarose gel illustrating the strong PCR signal observed for the reverse transcript (cDNA) of the BVMO3 gene candidate specifically during growth with 3-C_4-SPC. Also shown are positive controls for PCR with genomic DNA as the template (PCR control), and the RT-PCR positive controls (+) when testing for cDNA of a constitutively expressed gene (CtesDRAFT_PD5114; succinyl-CoA ligase *alpha* subunit). M, length marker in bp.

grown *C. testosteroni* KF-1 appeared to be labile, as observed previously (42), but supplementation of all buffers with glycerol, FAD, and $NADP^+$ (51) increased the activity and the stability. Under these conditions, the membrane-free soluble fraction exhibited a specific SAPMO activity of up to 0.12 μmol min^{-1} mg^{-1}, about 10-fold higher than observed previously (42) and, hence, closer to the calculated *in vivo* activity of 0.29 μmol min^{-1} mg^{-1} (42). Furthermore, the enzyme could be stored frozen without significant loss of activity, whereas about 50% of the activity was lost after 1 day at 4°C, no matter whether it was kept under air or nitrogen. After a first purification step (anion-exchange chromatography using Mono Q), about 50% of the total activity was lost, but the specific activity had increased 8-fold to 0.96 μmol min^{-1} mg^{-1}; a second Mono Q step at a higher pH was unsuccessful (no activity), as was a gel filtration step (no purification) (data not shown).

We used the partially purified native SAPMO (Mono Q fraction) to determine apparent K_m values for SAP and NADPH of 23 μM (±2 μM) and 6.3 μM (±1.2 μM) (means ± standard deviations), respectively, which confirmed a high affinity of the enzyme to the substrates (see below); NADH could not replace NADPH as the cosubstrate (42). Furthermore, the activity of SAPMO with some structural analogues of SAP was tested (see below): 4-hydroxyacetophenone (HAP), 4-aminoacetophenone, and 4-nitroacetophenone appeared to be good substrates, whereas neither cyclohexanone nor acetone was accepted, and these substrates were not inhibitory, as judged by normal activity upon the addition of SAP.

We anticipated a 60-kDa protein to be enriched in SAPMO active fractions compared to SAPMO inactive fractions when observed by denaturing SDS-PAGE, hence, a protein band representative of the monomer encoded by the BVMO3 candidate (predicted molecular mass, 59.8 kDa); the other three BVMO gene candidates encoded proteins with lower molecular masses (approximately 38 to 55 kDa). A valid band (at around 60 kDa) was observed, cut from the gel, and subjected to peptide fingerprinting-mass spectrometry (PF-MS) (data not shown), which attributed locus tag CtesDRAFT_PD5437 to it (score, >500, and coverage, >45%), i.e., the BVMO3 gene candidate (Fig. 2A). Three other prominent protein bands visible in the active fraction at lower molecular masses (approximately 58, 52 and 46 kDa) were also identified by PF-MS, but the fingerprints did not match those of any BVMO candidate genes (the locus tags identified were CtesDRAFT_PD5455, _PD3982, and _PD1824 for acetyl-CoA acetyltransferase, threonine synthase, and isocitrate dehydrogenase, respectively).

Heterologous expression of the SAPMO candidate gene and characteristics of the purified enzyme. The SAPMO candidate gene (BVMO3) was cloned and overexpressed in *E. coli* as a soluble, N-terminally His_6-tagged protein that could be purified via

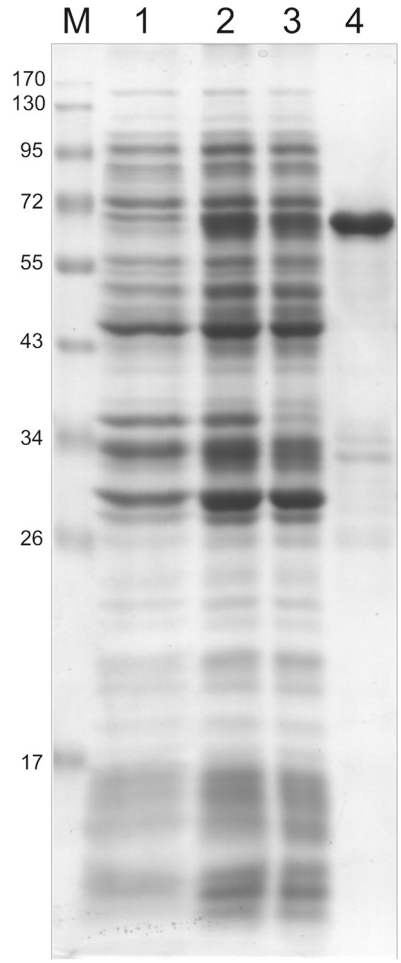


FIG 3 Analysis by SDS-PAGE of SAPMO (encoded by BVMO3 gene candidate) overexpression in *E. coli* and of the purification of the N-terminally His_6-tagged SAPMO protein. Lane 1, whole cells prior to IPTG induction (approximately 60 μg total protein); lane 2, crude extract 5 h after induction (100 μg protein); lane 3, soluble protein fraction after ultracentrifugation (80 μg protein); lane 4, protein fraction obtained from Ni-NTA purification (30 μg protein); lane M, molecular mass markers (kDa).

nitrilotriacetic acid (NTA)-agarose affinity chromatography (see Materials and Methods). SDS-PAGE (Fig. 3) confirmed that a prominent protein band at approximately 60 kDa had appeared in cell extracts after isopropyl-β-D-thiogalactopyranoside (IPTG) induction and that a single protein band at approximately 60 kDa (with negligible impurities at around 30 kDa) was obtained from the affinity chromatography step. The purified recombinant protein of *C. testosteroni* catalyzed an NADPH-dependent oxygenase reaction with SAP, as routinely assayed as the photometrical decrease of absorption of the cosubstrate NADPH. Furthermore, the disappearance of SAP and NADPH and the formation of SPAc and $NADP^+$ during the reaction were confirmed by HPLC analysis of samples taken during the reaction (see below); SAP-specific, NADPH-dependent oxygen consumption was confirmed with an oxygen electrode (42; data not shown). The activity of the recombinant enzyme showed an apparent K_m of 61.7 μM (and a V_{max} of 2.8 μmol min^{-1} mg^{-1}) for the reaction with SAP (Table 1); therefore, the K_m value was slightly higher than that determined for the native semipurified enzyme (see above). As illustrated by the kinetic parameters shown in Table 1, the recombinant SAPMO of *C. testosteroni* also catalyzed the conversion of several other aromatic ketones, during which the uncoupling rate (NADPH disappearance in the absence of a substrate) was negligible (0.03 μmol min^{-1} mg^{-1}). Interestingly, the conversion of phenylacetone (phenyl-2-propanone) occurred at a rate (and affinity) that was similar to the conversion of SAP (Table 1), whereas specific activities were lower for the other substrates, decreasing in the order 4-aminoacetophenone, 4-hydroxyacetophenone (HAP), 4-nitroacetophenone, 4-hydroxypropiophenone, acetophenone, and benzaldehyde (Table 1). However, the highest substrate affinity was determined for the reaction with HAP (K_m, 35.9 μM). Notably, progesterone, cyclohexanone, and acetone did not affect any measurable activity when tested under the conditions we used, and these substrates were not inhibitory as judged by normal activity upon the addition of SAP. The pH optimum of the SAPMO reaction, around pH 8, was determined to be broad. Furthermore, the temperature stability of SAPMO was determined as described for phenylacetone BVMO (PAMO) (32); however, the loss of about half of the activity after 1 h at a temperature of 30°C compared to the activity in the control reaction with enzyme that had been stored at 4°C indicated that SAPMO of *C. testosteroni* is not heat stable.

Purification, identification, and characterization of SPAc esterase. The SPAc esterase was highly active (1.5 μmol min^{-1} mg^{-1}) (Table 2) in comparison to the calculated *in vivo* activity (0.3 μmol min^{-1} mg^{-1}) (42). The enzyme was in the soluble fraction, and a three-step purification protocol (Table 2) was sufficient to obtain an essentially pure protein, as observed by SDS-PAGE (Fig. 4, lane 3); a further slight increase in purity was achieved by gel filtration (Fig. 4, lane 4) to obtain, finally, a 125-fold-purified homogenous protein with a yield of 12% (Table 2). The protein band was cut from the gel and analyzed by PF-MS, which identified locus tag CtesDRAFT_PD5438 (score, >800, and coverage, >60%) in the *C. testosteroni* KF-1 genome; thus, this is the esterase gene candidate *est3* (Fig. 2A). The predicted molecular mass of the gene product was 33.604 kDa, which matched the mass observed for the denatured protein by SDS-PAGE (Fig. 4). Furthermore, a native molecular mass of about 30 to 40 kDa was indicated during the calibrated gel filtration-chromatography run; thus, a monomeric structure of the enzyme is very likely. The purified enzyme catalyzed the conversion of the substrate SPAc to the products 4-sulfophenol and acetate in a 1:1:1 stoichiometry (not shown), which confirmed our earlier observations made with crude extracts (42). An apparent K_m value of 27 μM (±6) for SPAc

TABLE 2 Purification of SPAc esterase from *C. testosteroni* KF-1

Purification step[a]	Total protein (mg)	Total activity (μmol min^{-1})	Recovery (%)	Sp act (μmol min^{-1} mg^{-1})	Purification (fold)
Crude extract	85.8	127	100	1.5	1
Soluble fraction	64.0	106	83	1.7	1.1
ASP	41.0	95	75	2.3	1.6
Anion exchange	2.0	40	31	20.0	13.3
HIC	0.12	21	16	174.6	116
Gel filtration	0.08	15	12	188	125

[a] ASP, ammonium sulfate precipitation; HIC, hydrophobic interaction chromatography.

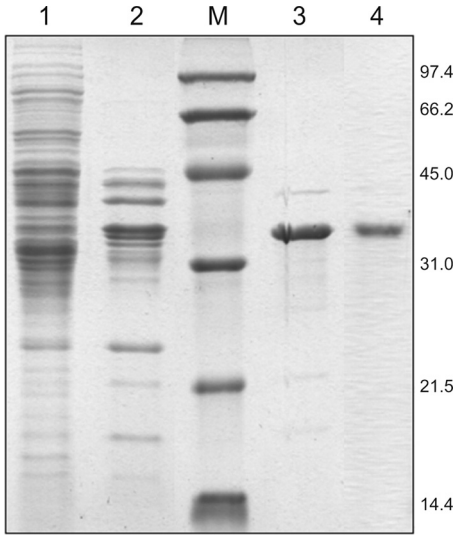


FIG 4 SDS-PAGE analysis of the different stages of purification of SPAc esterase of *C. testosteroni* KF-1. Lane 1, soluble protein fraction after ultracentrifugation; lane 2, active fraction after anion-exchange chromatography; lane M, molecular mass markers (with masses in kDa flanking the gel); lane 3, active fraction after hydrophobic interaction chromatography; lane 5, active fraction after gel filtration chromatography (from a different purification run).

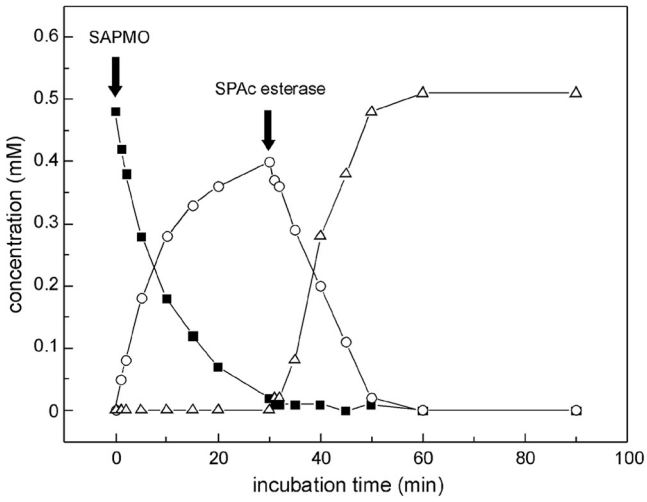


FIG 5 Reconstitution of the reaction sequence from SAP via SPAc to 4-sulfophenol using the purified SAPMO and SPAc esterase enzymes, as followed by HPLC-UV analysis of samples taken at intervals during the reactions. SAPMO (15 μg protein ml^{-1}) and SPAc esterase (0.7 μg protein ml^{-1}) were added sequentially (arrows) to a reaction mixture that contained 0.5 mM SAP (■) and 1.25 mM NADPH (not shown), which led to the formation of SPAc (○) and 4-sulfophenol (△), respectively. For clarity of the illustration, the data set showing a stoichiometric NADPH-to-$NADP^+$ conversion during the first reaction (determined by HPLC-UV) was omitted in this graph.

(V_{max} = 255 μmol min^{-1} mg^{-1}) was determined, showing a high affinity of the enzyme to its substrate. Finally, preincubation of the purified enzyme for 5 min at 30°C, 37°C, or 45°C did not result in a significant loss of activity (33), and 25% of the initial activity remained after 5 min of incubation at 60°C (see Discussion).

Reconstitution of the reaction sequence from SAP via SPAc to 4-sulfophenol. The two pure enzymes, SAPMO and SPAc esterase, were used to demonstrate quantitative NADPH-dependent formation of SPAc from SAP over time, followed by quantitative formation of 4-sulfophenol from SPAc and, hence, a stoichiometric conversion of SAP to 4-sulfophenol. Recombinant SAPMO was incubated with 0.5 mM SAP and 1.25 mM NADPH, and samples for HPLC-UV analysis were taken at intervals. After 30 min, purified esterase was added, and the reaction mixture was monitored further by HPLC-UV. The data obtained (Fig. 5) confirmed quantitative conversion of SAP to 4-sulfophenol through SAPMO and SPAc esterase, including the transient appearance of quantitative amounts of SPAc. Furthermore, the HPLC-UV data confirmed quantitative NADPH conversion to $NADP^+$ during the SAPMO reaction, and a quantitative formation of acetate was confirmed by gas chromatography in a sample taken after the SPAc esterase reaction (data not shown).

DISCUSSION

LAS is on the market and effectively degraded by microbes for over 50 years (36). It has also been known for many years that SPCs are intermediates in the degradation of LAS, but the microbial degradation pathway for short-chain SPCs resisted all attempts at elucidation (7, 10, 47, 53) until we established that SAP, SPAc, and 4-sulfophenol (Fig. 1) are intermediates in the 3-C_4-SPC degradation pathway in *C. testosteroni* KF-1 and that a BVMO and an esterase must be involved (42). Now, the first two enzymes (and genes) in the 3-C_4-SPC degradation pathway are known, a type I Baeyer-Villiger monooxygenase (EC 1.14.13.x) as SAPMO (locus tag CtesDRAFT_PD5437) and a carboxylesterase (EC 3.1.1.1) as SPAc esterase (locus tag CtesDRAFT_PD5438).

Research on BVMOs has progressed at an impressive pace in recent years (reviewed in reference 25), since an increasing number of BVMOs from various sources have been cloned and characterized, with several crystal structures available, for the aim of understanding their complex reaction mechanisms. In addition, there is interest in exploiting their diverse substrate preferences and extraordinary enantio-, regio-, and chemoselectivities for biotechnological applications, as well as in altering their characteristics through directed evolution (reviewed in reference 25). In the present study, we revealed an example of how bacteria are exploiting BVMOs to access a novel carbon and energy source for their growth; in this case, in *C. testosteroni* KF-1 for the utilization of the xenobiotic 3-C_4-SPC, derived from primary degradation of commercial LAS surfactants. Interestingly, it appeared that the identified SAPMO and SPAc esterase gene cluster (Fig. 2A) could have been mobilized recently from a different location into the genome of strain KF-1 as part of a catabolic transposon (e.g., see reference 50). First, the identified genes (together with other predicted catabolic genes, discussed below) are framed by two Tn*3* family transposase genes (Fig. 2A) with nearly identical sequences (99.9%) that, upon closer inspection (not shown), appear as complete insertion sequence (IS) elements of the IS*1071* family (i.e., the 110-bp inverted repeats, which are 99.8% identical to the archetypal IS*1071* of *C. testosteroni* BR60 [30]). IS*1071* elements have frequently been identified in close proximity to various xenobiotic degradation genes in environmental bacteria (4, 18, 30, 35) and have been shown to transpose at high frequencies in *C. testosteroni* strains (48). Second, homologous gene clusters of SAPMO and SPAc esterase genes framed by IS*1071* elements were not found in the other two *C. testosteroni* genomes available, those of strains S44 and CNB-2 (27, 54) (nor on plasmid pCNB1 of *C.*

testosteroni CNB-1 [26]). Given the wide substrate range of BVMOs (25), such as observed for SAPMO (Table 1), and given the anticipated wide substrate range of SPAc esterase (discussed below), it is easy to rationalize that such a multifunctional BVMO-esterase gene module mobilized via IS*1071*-mediated transposition would have been maintained in a bacterial genome if the genes became part of a novel biochemical pathway for exploiting novel growth substrates.

The sequence of SAPMO is typical of type I BVMOs, as it contains the BVMO fingerprint motif (FXGXXXHXXXWP) and two Rossman fold motifs (GXGXXG) for FAD and NADPH binding (12); the SAPMO sequence does not include the N-terminal extension (120 amino acids) of HAPMO-like type I BVMO sequences (see the sequence alignment in Fig. S1 in the supplemental material). SAPMO showed the highest BLASTp hits to BVMO candidate genes encoded in *Sorangium cellulosum* 56 (sce4944, 60% identical amino acids), in *Rhodopseudomonas palustris* BisB5 (RPD_1860, 58% identity), and surprisingly, in our LAS-degrading *Parvibaculum lavamentivorans* DS-1^T (Plav_0813, 58% identity) (43). With respect to cloned and well-characterized BVMOs, the highest homology of SAPMO was observed to phenylacetone BVMO (PAMO) (13) of *Thermobifida fusca* (57% identical amino acids) and steroid BVMO (STMO) (29) of *Rhodococcus rhodochrous* (55% identity). Cyclohexanone BVMO (CHMO) (46) and HAPMO sequences (20, 34) appeared to be more distantly related to SAPMO (around 45 and 30% identity, respectively). The observed phylogenetic relationship of SAPMO with the characterized type I BVMO PAMO described above appeared to be directly reflected in the substrate-dependent SAPMO activities determined (Table 1). PAMO appears to prefer substrates bearing phenyl groups, whereas other carbonylic compounds such as cyclohexanone are not accepted. SAPMO converted 4-amino- and 4-nitro-acetophenone with efficiencies similar to its conversion of HAP; acetophenone, 4-hydroxypropiophenone, and benzaldehyde were less effectively converted, and cyclohexanone and acetone were not substrates (3, 21). Furthermore, SAPMO did not convert progesterone, an observation that is in accordance with the substrate preference observed for PAMO (14). Finally, SAPMO exhibited a broad pH optimum, as typically observed for type I BVMOs, and in contrast to PAMO, was not heat stable (13). Overall, we identified and characterized a novel member of the type I BVMO enzyme family that plays a key role in a degradation pathway for a xenobiotic compound in bacteria.

The SPAc esterase sequence showed the highest BLASTp hits to sequences encoded in *Burkholderia xenovorans* LB400 (Bxe_A3606, 59% identical amino acids), *Cupriavidus pinatubonensis* JMP134 (Reut_B5462, 43% identity), and *Burkholderia* sp. CCGE1002 (BC1002_5819, 41% identity); the latter two homologs are, like the SPAc esterase gene, encoded in the immediate vicinity of a BVMO candidate gene. Furthermore, SPAc esterase showed 38% amino acid identity to HPAc esterase (ACA50464) in *Pseudomonas fluorescens* ACB, which is also coencoded with a corresponding BVMO gene, in this case HAPMO (28), as is the isofunctional, recently characterized (see below) carboxylester hydrolase in *P. putida* JD1 (termed PPE in reference 33), which shows 94% amino acid identity to HPAc esterase of strain ACB (33) (the PPE sequence was not deposited in GenBank). The latter gene (for PPE esterase) has been cloned and expressed and characterized as a purified enzyme (33). PPE hydrolyzed a wide range of aryl esters, cyclic esters, and tertiary alcohol esters with sterically more-demanding side groups at the quaternary carbon atom, making this type of carboxylester hydrolase useful for biotechnological applications (44). Besides the alpha/beta-hydrolase fold and catalytic triad (Ser/Asp/His), including the classical pentapepide (GDSAG) around the catalytic-site serine (5), the PPE esterase and SPAc esterase, as well as all of the other above-mentioned homologs, contain the additional GGGX motif (not shown). This motif has been recognized to represent an enlarged oxyanion pocket in the enzyme, allowing sterically more-demanding substrates to enter the active site than is possible for esterases with the more widespread GX motif and smaller active site (33, 17). In this study, we had only limited amounts of purified native SPAc esterase available; however, it will be interesting to see in future work whether the enzyme also exhibits such promiscuous behavior to a wide range of acetyl esters. Notably, SPAc esterase appeared to be rather temperature insensitive in comparison to PPE esterase, which suffered 66% loss of activity after only 5 min at 37°C and was completely inactivated at 45°C (33).

Access to a genome sequence and protein identification through PF-MS greatly facilitated this work, and this is particularly encouraging since questions remain regarding the nature of other important steps in the 3-C_4-SPC pathways in *C. testosteroni* KF-1. First, how is the acetyl-side chain removed from 3-C_4-SPC in strain KF-1 (Fig. 1; upper pathway) to yield SAP? This reaction sequence has been postulated to involve 3-C_4-SPC-CoA esters and reactions in analogy to short-chain fatty acid CoA oxidation, e.g., a beta-ketoacid lyase (discussed in reference 42). The SAPMO and SPAc esterase genes are clustered with predicted genes for acyl-CoA metabolism (Fig. 2A) (e.g., acyl-CoA synthetase, CoA-transferase, and acetyl-CoA acetyltransferase candidate genes), and we are currently exploring whether these genes play a role in the 3-C_4-SPC degradation pathway or not. Second, how is 4-sulfophenol converted to (desulfonated) central metabolites in strain KF-1? We found evidence for the involvement of 4-sulfocatechol as the substrate for a desulfonative *ortho*-ring cleavage pathway (40, 42, 45), as has been characterized in other bacteria (9, 15, 16); however, we have preliminary evidence to suggest that the pathway may also (or instead?) involve 2-hydroxyquinol (1,2,4-trihydroxybenzene) *ortho*-ring cleavage. Finally, the different biochemical strategies and pathways employed in other organisms that degrade other SPCs, e.g., for 4-C_6-SPC in *Delftia acidovorans* SPH-1 (40), remain completely unexplored, as are the enzymes of primary LAS degradation in *P. lavamentivorans* DS-1 (Fig. 1), but genome sequences for these organisms have been made available (Genomes Online Database [GOLD] identification numbers Gc00683 and Gc00631) (43). Hence, it is obvious that much more work lies ahead to completely uncover the metabolic pathways for SPCs and LAS in bacteria.

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SUPPLEMENTAL MATERIAL

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Two Enzymes of a Complete Degradation Pathway for Linear Alkylbenzenesulfonate (LAS) Surfactants: 4-Sulfoacetophenone Baeyer-Villiger Monooxygenase and 4-Sulfophenylacetate Esterase in *Comamonas testosteroni* KF-1

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TABLE S1. PCR primers.

primer name	gene locus tag (binding position [bp])	primer sequence (5' -3')	product length (bp)
bvmo1-for	CtesDRAFT_PD1901 (413–432)	GCGCCGGCTATTACGACCAC	640
bvmo1-rev [a]	CtesDRAFT_PD1901 (1052–1034)	CCGCCGAACCGCTTGAGAT	
bvmo2-for	CtesDRAFT_PD3136 (677-695)	TGCAGCGCGAACCCACCTA	556
bvmo2-rev [a]	CtesDRAFT_PD3136 (1232-1213)	TGCTCGCCGCGTTCTATCAG	
bvmo3-for	CtesDRAFT_PD5437 (51-71)	CTTCGCCGGCCTCTACCAACT	544
bvmo3-rev [a]	CtesDRAFT_PD5437 (594–577)	GGCGATCACGGGCACCAT	
bvmo4-for	CtesDRAFT_PD1705 (462-483)	CGTATCTCCCGAGCCGTTCAAA	498
bvmo4-rev [a]	CtesDRAFT_PD1705 (960–939)	ACCATAGCCCACCAGCCACAAG	
sucD-for	CtesDRAFT_PD5114 (118-137)	GCAGGCGTGAACCCCAAGAA	451
sucD-rev [a]	CtesDRAFT_PD5114 (568-550)	CGC CAC CGA TAC CGA CAG C	
est3-for	CtesDRAFT_PD5438 (88–106)	AGCGGCCCGATGTTGATGG	437
est3-rev [a]	CtesDRAFT_PD5438 (524–506)	GCGGGCTTGGCGTTGTTGT	
TOPO-for [b]	CtesDRAFT_PD5437 (1–20)	CACC**ATG**AGCAATTCAACCACCCG	1616
TOPO-rev [c]	CtesDRAFT_PD5437 (1616-1596)	TT**TCA**GGCCTTCGCAAAGCCC	

[a] primer used for reverse-transcription reaction.

[b] the adaptor-sequence for directional TOPO-cloning is underlined in the primer sequence and the start codon in bold letters.

[c] the stop codon is indicated in bold letters.

Fig. S1 [following pages]. Alignment of the amino-acid sequences of the four type-I BVMO candidate genes (*bvmo1 – bvmo4*) in the genome of *C. testosteroni* KF-1 and of four characterized type-I BVMOs for comparison. The characterized type-I BVMOs included in the alignment are phenylacetone monooxygenase of *Thermobifida fusca* YX (PAMO [Q47PU3]), steroid monooxygenase of *Rhodococcus rhodochrous* (STMO [BAA24454]), and 4-hydroxyacetophenone monooxygenase of *Pseudomonas fluorescens* ACB (HAPMO [AAK54073]) and of *Pseudomonas putida* JD1 (HAPMO [ACJ37423]). The characteristic 'BVMO-fingerprint' sequence motif (see ref. below) is indicated in red and the two Rossman-fold motifs for FAD and NADPH-binding are indicated in blue; other characteristic flavoprotein sequence motifs are also indicated (in black). Note that the sequence of the *bvmo4* candidate contains two substitutions in the BVMO-fingerprint motif (FXGXXXHXXX**Y-V** instead of FXGXXXHXXX**W**-(**P/D**)).

Reference for legend of Fig. S1: **Fraaije MW, Kamerbeek NM, van Berkel WJ, Janssen DB.** 2002. Identification of a Baeyer-Villiger monooxygenase sequence motif. FEBS Lett. **518:** 43-47.

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bvmo3/SAPMO [CtesDRAFT_PD5437]      ------------------------------------------------------------
bvmo1 [CtesDRAFT_PD1901]            ------------------------------------------------------------
bvmo2 [CtesDRAFT_PD3136]            ------------------------------------------------------------
bvmo4 [CtesDRAFT_PD1705]            ------------------------------------------------------------
PAMO  [Q47PU3]                      ------------------------------------------------------------
STMO  [BAA24454]                    ------------------------------------------------------------
HAPMO [AAK54073]                      1 MSAFNTTLPSLDYDDDTLREHLQGADIPTLLLTVAHLTGDLQILKPNWKPSIAMGVARSG
HAPMO [ACJ37423]                      1 MRTYNTTLASLECDDETLRAHLQGADIPTLLLTVAHLTGDLNVLKPAWKPVVAMGVAHSG

bvmo3/SAPMO_[CtesDRAFT_PD5437]      ------------------------------------------------------------
bvmo1 [CtesDRAFT_PD1901]            ------------------------------------------------------------
bvmo2 [CtesDRAFT_PD3136]            ------------------------------------------------------------
bvmo4 [CtesDRAFT_PD1705]            ------------------------------------------------------------
PAMO  [Q47PU3]                      ------------------------------------------------------------
STMO  [BAA24454]                    ------------------------------------------------------------
HAPMO [AAK54073]                     61 MDLETEAQVREFCLQRLIDFRDSGQPAPGRPTSDQLHILGTWLMGPVIEPYLPLIAEEAV
HAPMO [ACJ37423]                     61 MTPEVEAEVREDCLQKLLAFRDSGLPVPARPSSEQLHALGTWLMGPVIEPYLPLVAEELV

                                                          GXGXXG                          GG
bvmo3/SAPMO_[CtesDRAFT_PD5437]      1 MSNSTTR------------QVDLVIVGAGFAGLYQLHRARGLGLK--TQVLEAGDGVGG
bvmo1 [CtesDRAFT_PD1901]            1 -----------------MAQHFDFLIIGAGISGIGAARHLREQFADRCLAILEAMDGFGG
bvmo2 [CtesDRAFT_PD3136]            1 -------------------MKYDVIVIGCGMSGILAGIHLKNSGKK--FIILEKAKTLGG
bvmo4 [CtesDRAFT_PD1705]            1 -----------------MSFHDVIIIGAGQAGLSVAYFLRRTNLS--VLLLDAEEVGGG
PAMO  [Q47PU3]                      1 MAGQTTVDSRRQPPE-----EVDVLVVGAGFSGLYALYRLRELGRS--VHVIETAGDVGG
STMO  [BAA24454]                    1 MNGQHPRSVVTAPDATTGTTSYDVVVVGAGIAGLYAIHRFRSQGLT--VRAFEAASGVGG
HAPMO [AAK54073]                  121 TAEEDLRAPRWHKDHVASGRDFKVVIIGAGESGMIAALRFKQAGVP--FVIYEKGNDVGG
HAPMO [ACJ37423]                  121 TAEEDPRAPRWHKDHVAAGREFKVVIIGAGVSGMIAALRFKQAGVP--FVVYEKGTDVGG
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bvmo3/SAPMO [CtesDRAFT_PD5437]   46 TWFWNRYPGARCDVESLDYSYSFSDELQQEWNWSERYAPQPEILRYINHVADRFDLRKDI
bvmo1 [CtesDRAFT_PD1901]         44 TWWTHRYPGARSDSDLFTYGYGFKPWT------GNAIATADEIRHYLAETIEENGLAPLI
bvmo2 [CtesDRAFT_PD3136]         40 TWRDNTYPGLTCDVPSHAYTYSFEPNP----EWSRVLPPGAEIQQYFEGVFLKYGIVDFS
bvmo4 [CtesDRAFT_PD1705]         41 AWQHGWDSLRLFSPASWSSIAGWLMPP-----SGEQYPSRDHVVDYLR----KYETRYEF
PAMO  [Q47PU3]                   54 VWYWNRYPGARCDIESIEYCYSFSEEVLQEWNWTERYASQPEILRYINFVADKFDLRSGI
HAPMO [AAK54073]                179 TWRENTYPGCRVDINSFWYSFSFARG-----IWDDCFAPAPQVFAYMQAVAREHGLYEHI
HAPMO [ACJ37423]                179 TWRENTYPGCRIDINSFFYSFSFARS-----TWDDCFAPGPQVFSYMQAVARDKGLYDHI
STMO  [BAA24454]                 59 VWYWNRYPGARCDVESIDYSYSFSPELEQEWNWSEKYATQPEILAYLEHVADRFDLRRDI

bvmo3/SAPMO [CtesDRAFT_PD5437]  106 QFNSRVKSATFDESRVRWTVETEAGE-----KFDAQYLIMATGCLSIPQ--PPKFKGLEG
bvmo1 [CtesDRAFT_PD1901]         98 RYGHKVQSARWSSEDRRWTLEVTHSGSDEVQTFTTGFLWVCAGYYDHGRGYVPEWPTLKD
bvmo2 [CtesDRAFT_PD3136]         96 QFDTEVTRAEWTG--EAWTLQDQHGK-----QYQAKVVVAATGVLHHPN--YPQIKGLEE
bvmo4 [CtesDRAFT_PD1705]         92 LIERPVLVTSVEPTEQGFQVNAGATS------WHSRAVVCATGTWRNPF--VPEVEGMTS
PAMO  [Q47PU3]                  114 TFHTTVTAAAFDEATNTWTVDTNHGD-----RIRARYLIMASGQLSVPQ--LPNFPGLKD
STMO  [BAA24454]                119 RFDTRVTSAVLDEEGLRWTVRTDRGD-----EVSARFLVVAAGPLSNAN--TPAFDGLDR
HAPMO [AAK54073]                234 RFNTEVSDAHWDESTQRWQLLYRDSEGQ--TQVDSNVVVFAVGQLNRPM--IPAIPGIET
HAPMO [ACJ37423]                234 QFNTEVTDAHWDEDRQRWQLLCRDSAGQ--TRVDSNVVVFAVGQLNRPM--IPAIPGKET

                                     FXGXXXHXXXW                GXGXXG
bvmo3/SAPMO [CtesDRAFT_PD5437]  159 FKGKWYHSADWPREGVDFTGKRVGLIGTGSSGVQMVPVIAEQAAHLTVFQRTANFSVPAQ
bvmo1 [CtesDRAFT_PD1901]        158 FKGQVIHPQHWPQE-LDYEGKRIVVIGSGATAATLIPSLAPDAGHVTMLQRSPTFFLPAS
bvmo2 [CtesDRAFT_PD3136]        147 FGGNLIHSARWDHS-IPLDGKRIAIIGTGSTGVQIVSALASR-AKVRHFQRTAQWIFPVE
bvmo4 [CtesDRAFT_PD1705]        144 FKGQQMHSAQYVSP-EPFKGKRVMVVGGGNSGAQILAEVSLVAESTTWVTLEPPAFLPDE
PAMO  [Q47PU3]                  167 FAGNLYHTGNWPHEPVDFSGQRVGVIGTGSSGIQVSPQIAKQAAELFVFQRTPHFAVPAR
STMO  [BAA24454]                172 FTGDIVHTARWPHDGVDFTGKRVGVIGTGSSGIQSIPIIAEQAEQLFVFQRSANYSIPAG
HAPMO [AAK54073]                290 FKGPMFHSAQWDHD-VDWSGKRVGVIGTGASATQFIPQLAQTAAELKVFARTTNWLLPTP
HAPMO [ACJ37423]                290 FQGPAFHSAQWDHN-VDWSGKRVAVIGTGASAAQFIPQLAKTAADLKVFARTTNWLLPTP
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bvmo3/SAPMO [CtesDRAFT_PD5437]  219 NEPLSEETLAQVKANYAERRALGREAVTGVFLMANDKSAVEVSDEERLKEFEFRWRGAGG
bvmo1 [CtesDRAFT_PD1901]        217 EHPLVP------LLKPLNLPEDWYHEIMRRAFIARTDEIVQTSRQHPEAMRAFLLG----
bvmo2 [CtesDRAFT_PD3136]        205 NPAFSEEQRAEFRSNRDLLVYLQRE-PTYMANVERFTEGVLDPDSEQIQEIQKICQ----
bvmo4 [CtesDRAFT_PD1705]        203 VDGRVLFERATARWQ-----------------------------------------------
PAMO  [Q47PU3]                  227 NAPLDPEFLADLKKRYAEFREESRNTPGGTHRYQGPKSALEVSDEELVETLERYWQ--EG
STMO  [BAA24454]                232 NVPLDDATRAEQKANYAERRRLSRESGGGSPHRPHPKSALEVSEEERRAVYEERWK--LG
HAPMO [AAK54073]                349 DLHEKISDSCKWLLAHVPHYSLWYRVAMAMPQSVGFLEDVMVDVGYPPTELAVSAR----
HAPMO [ACJ37423]                349 DLHEKISDSCKWLLANLPNYSLWYRATKVMPQSVGFLEDVVVDVDYPPTEVAVSAR----

bvmo3/SAPMO [CtesDRAFT_PD5437]  279 GFRMLRAFNDLLRNPVSNKHAADFARSKIRAVVKDPVKADLLCPKEDLPFGTKRLCVDTN
bvmo1 [CtesDRAFT_PD1901]        267 ----------EIRSHLPEG---------------FDIDRHFNPGY-RPWQQRIAVVPNG
bvmo2 [CtesDRAFT_PD3136]        260 ---------DNLDASVT----------------DAALRQKLQPHY--RAGCKRLIYSPD
bvmo4 [CtesDRAFT_PD1705]        218 ------------------------------------ALQEGKDPEN-LPGGFGEIVMVPP
PAMO  [Q47PU3]                  285 GPDILAAYRDILRDRDANERVAEFIRNKIRNTVRDPEVAERLVPKG-YPFGTKRLILEID
STMO  [BAA24454]                290 GVLFSKAFPDQLTDPAANDTARAFWEEKIRAVVDDPAVAELLTPKD-HAIGAKRIVTDSG
HAPMO [AAK54073]                405 --------NDRLRQDISAWMEPQFADR--------PDLREVLIPDS--PVGGKRIVRDNG
HAPMO [ACJ37423]                405 --------NDQLRRDICAWMEPQFADR--------PDLRNVLIPDS--PVGSKRIIRDNG

                                                                ATG
bvmo3/SAPMO [CtesDRAFT_PD5437]  339 -YYETFNRDNVDLVDVKANPITEVTPNGLRTTT-SEHELDAIIFATGFDAMTGALLSMDI
bvmo1 [CtesDRAFT_PD1901]        300 DFFQSMREGKASVVT---DTIERFDGSGIQLTGGGHLDADIIVTATGFNLKR-------F
bvmo2 [CtesDRAFT_PD3136]        292 -YYQAIQQPDSELIT---EAIAQVEKSGVRTSDGVLHEVDIIVLATGFKTDR-YVRPMHV
bvmo4 [CtesDRAFT_PD1705]        241 -VLDARNRGVLHSVG----PFARLSAEGAQWADGSAKAFDAIIWCTGFRPALQPLESLGL
PAMO  [Q47PU3]                  344 -YYEMFNRDNVHLVDTLSAPIETITPRGVRTSE-REYELDSLVLATGFDALTGALFKIDI
STMO  [BAA24454]                349 -YYETYNRDNVELVDLRSTPIVGMDETGIVTTG-AHYDLDMIVLATGFDAMTGSLDKLEI
HAPMO [AAK54073]                447 TWISTLKRDNVSMIR---QPIEVITPKGICCVDGTEHEFDLIVYGTGFHASK-FLMPINV
HAPMO [ACJ37423]                447 TWISTLKRDNVSMIR---QPIEAIRPTGICCIDGIVHEFDLIVYGTGFHASK-FLMPINI
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bvmo3/SAPMO [CtesDRAFT_PD5437]  397 RGTGGQTLREKWAAG-PRTYLGVSIAGFPNMFVIAGPGS-PSVLSNVVHSIETHVNWLSD
bvmo1 [CtesDRAFT_PD1901]        350 GGIAFSVDDAPVDFRERISYRGVMIEGLPNMAYTQGYFR-----SSWTLRCDLVCDWVCR
bvmo2 [CtesDRAFT_PD3136]        347 KGLNAATLEHAWSDV-PTAYKSISVPGFPNFYFMNGPTS-PVGNFSLIDTSEMQWGYISQ
bvmo4 [CtesDRAFT_PD1705]        296 VNDSKVAVEGTRASD----RPSLWLVGYG---EWTGPAS-----ATLIGVMRTARDTASE
PAMO  [Q47PU3]                  402 RGVGNVALKEKWAAG-PRTYLGLSTAGFPNLFFIAGPGS-PSALSNMLVSIEQHVEWVTD
STMO  [BAA24454]                407 VGRGGRTLKETWAAG-PRTYLGLGIDGFPNFFNLTGPGS-PSVLANMVLHSELHVDWVAD
HAPMO [AAK54073]                503 TGRDGVALHDVWKGDDARAYLGMTVPQFPNMFCMYGPNTGLVVYSTVIQFSEMTASYIVD
HAPMO [ACJ37423]                503 TGRDGLALREVWKDDDARAYMGMTVPGFPNMFCMYGPNTGLVVYSTIIQFSEFTATYIVD

bvmo3/SAPMO [CtesDRAFT_PD5437]  455 LLERTRAQGIQRIEAEQAHEDKWVDHVN--EAANQTLYPVGNSWYVGANMPGKPRVFMPY
bvmo1_[CtesDRAFT_PD1901]        405 LLAHMREQGHAEVRPTVAAADAGMQRLS--WIEADNFNAGYVLRAQDAMFGQGDRQPWRH
bvmo2_[CtesDRAFT_PD3136]        405 LIERGEQAGVAGLSARPEALAQY-DRERLEAAKGSVFGSGCSSWYLDR-----NGVPNTW
bvmo4_[CtesDRAFT_PD1705]        344 IAQHLSRSE-------------------------------------------------
PAMO_[Q47PU3]                   460 HIAYMFKNGLTRSEAVLEKEDEWVEHVN--EIADETLYPMTASWYTGANVPGKPRVFMLY
STMO_[BAA24454]                 465 AIAYLDARGAAGIEGTPEAVADWVEECR--NRAEASLLNSANSWYLGANIPGRPRVFMPF
HAPMO_[AAK54073]                563 AVRLLLEGGHQSMEVKTPVFESYNQRVDEGNALRAWGFSKVNSWYKNS----KGRVTQNF
HAPMO_[ACJ37423]                563 AVRLLLEGGHQSMEIKAQVFESYNQRVDQANGLRAWGFSRVNSWYKNS----KGRVTQNF

bvmo3/SAPMO [CtesDRAFT_PD5437]  513 VAGVPAYRRIIEGVAEKDYEGFAKA----
bvmo1 [CtesDRAFT_PD1901]        463 DLEYAEERVVLPAASLQDDALAYR-----
bvmo2 [CtesDRAFT_PD3136]        459 PWSQSRFRQEMSKPVWQDYIHHQTEELAA
bvmo4 [CtesDRAFT_PD1705]            ----------------------------
PAMO  [Q47PU3]                  518 VGGFHRYRQICDEVAAKGYEGFVLT----
STMO  [BAA24454]                523 LGGFGVYREIITEVAESGYKGFAILEG--
HAPMO [AAK54073]                619 PFTAVEFWQRTHSVEPTDYQLG-------
HAPMO [ACJ37423]                619 PFNAVEFWHRTHEVQATDYCLS-------
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