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Degradation of Alkyl Methyl Ketones by *Pseudomonas veronii* MEK700[▽]

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***Pseudomonas veronii* MEK700 was isolated from a biotrickling filter cleaning 2-butanone-loaded waste air. The strain is able to grow on 2-butanone and 2-hexanol. The genes for degradation of short chain alkyl methyl ketones were identified by transposon mutagenesis using a newly designed transposon, mini-Tn*5495*, and cloned in *Escherichia coli*. DNA sequence analysis of a 15-kb fragment revealed three genes involved in methyl ketone degradation. The deduced amino acid sequence of the first gene, *mekA*, had high similarity to Baeyer-Villiger monooxygenases; the protein of the second gene, *mekB*, had similarity to homoserine acetyltransferases; the third gene, *mekR*, encoded a putative transcriptional activator of the AraC/XylS family. The three genes were located between two gene groups: one comprising a putative phosphoenolpyruvate synthase and glycogen synthase, and the other eight genes for the subunits of an ATPase. Inactivation of *mekA* and *mekB* by insertion of the mini-transposon abolished growth of *P. veronii* MEK700 on 2-butanone and 2-hexanol. The involvement of *mekR* in methyl ketone degradation was observed by heterologous expression of *mekA* and *mekB* in *Pseudomonas putida*. A fragment containing *mekA* and *mekB* on a plasmid was not sufficient to allow *P. putida* KT2440 to grow on 2-butanone. Not until all three genes were assembled in the recombinant *P. putida* was it able to use 2-butanone as carbon source. The Baeyer-Villiger monooxygenase activity of MekA was clearly demonstrated by incubating a *mekB* transposon insertion mutant of *P. veronii* with 2-butanone. Hereby, ethyl acetate was accumulated. To our knowledge, this is the first time that ethyl acetate by gas chromatographic analysis has been definitely demonstrated to be an intermediate of MEK degradation. The *mekB*-encoded protein was heterologously expressed in *E. coli* and purified by immobilized metal affinity chromatography. The protein exhibited high esterase activity towards short chain esters like ethyl acetate and 4-nitrophenyl acetate.**

The production of 2-butanone in 1992, as an example, was 840,000 tons/year worldwide, and the demand is still increasing (30). It is one of the most commonly used organic solvents for paints and varnishes and, therefore, is observed as the major component in the waste air of plants of the paint and lacquer industries. Waste air of this type is often purified by the use of biological treatment systems, i.e., biotrickling filter or biofilter (16, 46). The performance of such filters is dependent on the kind and activity of organisms performing the degradation reactions.

A common microbial strategy for aerobic degradation of ketones is the insertion of a single oxygen atom between the keto group and the adjacent carbon by a reaction equivalent to a chemical Baeyer-Villiger reaction. So-called Baeyer-Villiger monooxygenases (BVMOs) are involved in degradation of aliphatic, alicyclic, and aryl ketones and in combination with alcohol dehydrogenases and alkane hydroxylases in degradation of secondary alcohols, linear and cyclic alkanes, etc. (37). This type of microbial reaction was reported as early as 1953 with the removal of the C-17 side chain of progesterone (22), but only in 1969 was it proposed by Forney and Markovetz (19) that the degradation of 2-tridecanone by some *Pseudomonas* species involves a oxygenase which converts the 2-tridecanone into an ester analogous to a Baeyer-Villiger reaction and that the ester is hydrolyzed to acetate and a primary alcohol. Both oxygenase and esterase activities were found in crude extracts of the *Pseudomonas* spp. strains grown on that substrate, but the genes were never cloned. Later, *Nocardia* sp. strain LSU-169 was isolated which used a similar process to produce ethyl acetate and ethanol as intermediates during growth on 2-butanone as carbon source. The protein capable of hydrolyzing ethyl acetate was isolated, a homodimeric protein with a molecular weight of 39,500 for the subunit (17). It showed very high activity with ethyl acetate and 4-nitrophenylacetate as substrates (about 1,000 U/mg of protein). Again, the gene was never cloned. Methyl ketones and secondary alcohols were produced from mycobacterial species grown on the gaseous alkanes propane, butane, pentane, and hexane (35). After adaptation to the alkanes, they were also able to grow on the corresponding methyl ketones. Obviously, the alkanes were the inducers for expression of genes involved in degradation of the alkanes via subterminal oxidation. The subterminal oxidation of alkanes followed by a Baeyer-Villiger reaction seems to be exceptional, since most alkanes are degraded by terminal oxidation, as seen in the metabolism of butane by *Pseudomonas butanovora*. Here, butane is initially oxidized at the terminal carbon atom to produce 1-butanol, which is further oxidized to butyrate (5). Therefore, it is not surprising that most Baeyer-Villiger monooxygenases were found in degradation of alicyclic compounds, like cyclohexane or cyclohexanone, or the bicyclic campher. In particular, the cyclohexanone monooxygenase from *Acinetobacter* has been studied in great detail (42). Generally, they are flavoproteins with a requirement for re-

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duced NADPH and dioxygen (20). Some exhibit a very broad substrate specificity and produce chiral products with high enantiomeric excess, which makes them interesting for the chemical and pharmaceutical industries (37).

In the present study we aimed to study the microbiology and genetics of 2-butanone degradation. A *Pseudomonas* sp. strain degrading 2-butanone was isolated from a biotrickling filter treated with 2-butanone-polluted waste air. The objective of the present work was to identify the degradative genes actually expressed during 2-butanone metabolism and to elucidate the degradation pathway in this strain.

MATERIALS AND METHODS

Bacterial strains and culture conditions. For standard molecular biology techniques like DNA cloning, plasmid preparations, and DNA sequencing, *E. coli* JM109 (47) was used as host. Heterologous gene expression was done in *E. coli* JM109 and *P. putida* KT2440 (44). *Pseudomonas* sp. strain MEK700 was isolated from a bioreactor during a diploma thesis performed by Thorsten Müller at our institute (University of Stuttgart). The bioreactor was initially inoculated with active sludge from the water treatment plant near the institute and incubated with 2-butanone-polluted waste air for 24 days. The 16S RNA of this new strain was sequenced by Jorge Lalucat, Universidad de las Islas Baleares, Palma de Mallorca, Spain, and found to be 100% identical to the one of *P. veronii* strain INA06 (1). According to this close relationship, the strain was called *P. veronii* MEK700.

Mobilization of plasmids from *E. coli* to *P. putida* and *P. veronii* via conjugation was obtained with *E. coli* S17-1 (43). The strains were usually grown in LB liquid medium and on LB agar plates (36), with *E. coli* at 37°C and the *Pseudomonas* strains at 30°C. The minimal medium (MM) was mixed from several separately sterilized solutions. To 940 ml of distilled water, 50 ml phosphate buffer (per liter, 20 g KH_2PO_4, 70 g $Na_2HPO_4 \cdot 2H_2O$, pH 7.1), 10 ml salt solution (SL100; per liter, 100 g $(NH_4)_2SO_4$, 1 g $MgSO_4 \cdot 7H_2O$, 1 g NH_4Fe^{3+} citrate), 100 ml trace element solution (SL6; see below), and 1 ml calcium nitrate solution (CA 1000; per liter, 50 g $Ca(NO_3)_2 \cdot 7H_2O$) were added. The trace element solution (SL6) contained (per liter) 100 mg $ZnSO_4 \cdot 7H_2O$, 30 mg $MnCl_2 \cdot 4H_2O$, 300 mg H_3BO_3, 200 mg $CoCl_2 \cdot 6H_2O$, 10 mg $CuCl_2 \cdot 2H_2O$, $NiCl_2 \cdot 2H_2O$, and 30 mg $Na_2MoO_4 \cdot 2H_2O$. For agar plates, 15 g agar (Merck) was added to 940 ml distilled water and autoclaved separately. Glucose was added to a 0.2% (wt/vol) final concentration, and 2-butanone was supplied as vapor in a sealed exsiccator (0.1 ml in a 1.5-liter exsiccator). 2-Hexanol and 1-octanol were added to the agar medium at a final concentration of 2 mM, and the plates were inoculated with bacteria and incubated in an exsiccator sealed with petrolatum (Baysilone 35 g; GE Bayer Silicones). Antibiotics were added to liquid media and agar plates when necessary to select for plasmids and transconjugants (ampicillin, 100 μg ml^{-1}; kanamycin, 40 μg ml^{-1}; nalidixic acid, 10 μg ml^{-1}).

DNA manipulation techniques. Standard procedures were used for restriction enzyme analysis, ligation, and transformation of *E. coli* (40). For cloning of mini-Tn*5495* transposon insertions in *P. veronii*, 1 μg chromosomal DNA was digested overnight with 20 U of the restriction enzyme, and the DNA was purified with GFX PCR DNA and band purification kit columns (GE Healthcare) and ligated in a volume of 100 μl at room temperature overnight to support circularization of the fragments. The DNA was concentrated by isopropanol precipitation and resuspended in 20 μl Tris-EDTA buffer, pH 8.0 for transformation. PCRs were performed in 100-μl reaction mixtures using the Expand high-fidelity PCR kit (Roche Applied Science). Plasmids were prepared with the QIAprep spin miniprep kit (QIAGEN GmbH). Genomic DNA was prepared by $CsCl_2$ dye buoyant density centrifugation according to the methods of Sedlmeier and Altenbuchner (41).

DNA sequencing and annotation. DNA sequences were obtained from a Pharmacia automated laser fluorescence sequencer, the AutoRead sequencing kit and the Repro Gel Long system came from Amersham Pharmacia Biotech (now GE Healthcare), and oligonucleotides were obtained from MWG Biotech. The sequences were aligned with LASERGENE 99 software (DNASTAR). Annotations were done with the BLAST programs (4) at the electronic mail server from the National Center for Biotechnology Information, Bethesda, MD.

Plasmid constructions. The plasmid pCro2a was constructed from pJOE867 (2) and pUTminiTn*5*Km-1 (13). First, the plasmid pJOE867 was cut by HindIII, and the ends were filled in by Klenow polymerase and deoxynucleoside triphosphates and ligated to remove the HindIII site (pCro1). The new plasmid pCro1 was cut with BamHI and EcoRI and the ends filled in again by Klenow polymerase. The fragment was ligated to pUTminiTn*5*Km-1, which was cut before with SfiI, and the protruding ends were filled in by Klenow polymerase. The new plasmid, which had the Km-1 region replaced by the pCro1 derivative, was called pCro2a, and the new mini-Tn*5* derivative was called mini-Tn*5495*.

The broad-host-range positive selection vector pJOE4928.1 was constructed from pBBR1MCS-2 (33). First, the plasmid was cut with AgeI/NcoI and the *lacPOZ*α fragment replaced by the two complementary oligonucleotides s3902 (5′-CAT GTT TAA ATC TAG ATT TAA AT) and s3903 (5′-CCG GAT TTA AAT CTA GAT TTA AA) to give pJOE4908.1. From pIC20E (15) the polylinker was removed by EcoRI and then the *lacPOZ*α fragment PCR amplified with the oligonucleotides p3705 (5′-AAG ATA TCA GAT CTT ACG CGT ACG AGC TCG AAT TCA TCG ATC ATT CGC CAT TCA GGC T) and p3706 (5′-AAG ATA TCA GAT CTT ACG CGT ACG AGC TCG AAT TCA TCG ATC CAA TAC GCA AAC CGC CT). The PCR fragment was cleaved with EcoRV and inserted into pJOE875 (3), which was cleaved with HindIII and the ends filled in by Klenow polymerase. From here the *lacPOZ*α fragment was cut out again with XbaI together with the two inverted T4 transcription terminator sequences and inserted into the XbaI site of pJOE4908.1 to give pJOE4928.1. The plasmid pJOE5293.1 was obtained by amplification of *mekA* and *mekB* from *P. veronii* MEK700 chromosomal DNA with the primers s4122 (5′-AAA AAG AGC TCG GGC GCT CGT AAC GCC AAA) and s4140 (5′-AAA AAA GAG CTC GGA CAT TCA ATC ATC AGG ATA). The amplified fragment was cut with SacI and inserted into pJOE4928.1 cleaved with the same enzyme. For plasmid pJOE5332.10 *mek4*, *mekB*, and *mekR* were amplified with the primers s4122 (see above) and s4151 (5′-AAA AAA GAG CTC GGA GAC CGG GCT CTT CCT A) and the fragment inserted into pJOE4928.1 via SacI digestion of the vector and PCR fragment.

Protein purification by immobilized metal affinity chromatography (IMAC). An overnight culture of JM109 pJOE5358.1 in LB liquid medium was diluted 100-fold in 50 ml LB plus ampicillin. After 2 h growth at 37°C the culture was shifted to 28°C and the cells induced by adding 0.2% L-rhamnose. The cells were grown for 6 h on a shaker and harvested by centrifugation (Sorvall SS34; 10 min, 7,000 rpm, 4°C), washed with cold 0.1 M $NaPO_4$, pH 7, and resuspended in the same buffer (20 ml). The cells were disrupted in a high-pressure homogenizer (EmulsiFlex-C5; Avestin, Canada), which was chilled on ice, and cell debris was removed by centrifugation (Sorvall SS34; 20,000 rpm, 30 min, 4°C). All further steps were done at room temperature. The supernatant (18 ml) was added to 1.5 ml of Ni-nitrilotriacetic acid (NTA)–agarose resin (QIAGEN GmbH) in a column by using gravity flow. The resin was washed 12 times with 10 ml washing buffer each time (50 mM potassium phosphate, 300 mM NaCl, pH 7.0), and the bound protein was eluted with 2.5 ml elution buffer (50 mM potassium phosphate, 200 mM imidazole, pH 7.0) applied in aliquots of 500 μl. Fractions with high protein content (fractions 2 to 4) were combined and applied to a NAP10 column (GE Healthcare) to remove the imidazole. The enzyme was stored at −20°C by adding an equal volume of 85% glycerol.

Protein electrophoresis and protein determination. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done according to the methods of Laemmli (34). Protein concentrations were determined by the method of Bradford (8) using the Bio-Rad protein assay dye reagent and bovine serum albumin as standard.

Mating of *E. coli* S17-1/pCro2a and *P. veronii* MEK700 and screening of transposon mutants affected in 2-butanone degradation. Overnight cultures of *E. coli* S17-1 and *P. veronii* at 30°C in LB with kanamycin and without kanamycin, respectively, were 100-fold diluted in LB liquid broth and separately grown at 30°C for about 6 h. A 50-μl aliquot of each culture was added to 5 ml LB liquid and mixed, and 0.1 ml was dropped onto an LB agar plate, which was incubated overnight at 30°C. The cells were scraped off the plate with 5 ml minimal medium without carbon source. Various dilutions were then plated on agar plates with minimal medium containing 0.2% glucose, 50 μg/ml kanamycin, and 10 μg/ml nalidixic acid (counterselection against the donor *E. coli*; the recipient *P. veronii* tolerates up to 10 μg/ml nalidixic acid). The plates were incubated overnight at 30°C. Colonies appearing on the plates were toothpicked onto MM agar plates with glucose and antibiotics, 100 colonies per plate, and again incubated at 30°C overnight. From the master plates the colonies were replica plated on four MM agar plates with antibiotics, one without a carbon source, one with 2-hexanol, one with 1-octanol and, as a control, again on MM plates with antibiotics and glucose. The plates without carbon source were brought into an exsiccator (1.5-liter volume) and 0.1 ml 2-butanone was provided in an open glass beaker. The exsiccator was sealed with petrolatum and again incubated at 30°C for up to 2 days.

Esterase activity. Esterase activity was determined colorimetrically with 4-nitrophenyl compounds or in a pH stat (Metrohm, Herisau, Switzerland) at 30°C. In the pH stat the ester was added to a final concentration of 100 mM to 10 ml

of 0.1 mM sodium phosphate buffer, pH 7.0. Adding purified enzyme started the reaction. The pH was kept constant by titration with 1 mM NaOH. One unit is defined as the amount of enzyme that liberates 1 μmol acid per ml per min under assay conditions. With 4-nitrophenyl acetate and 4-nitrophenyl butyrate, the enzyme activity was determined in a spectrophotometer. The assay was done in 1 ml of 100 mM sodium phosphate buffer, pH 7.0, with 1 mM 4-nitrophenyl acetate or 4-nitrophenyl butyrate (stock solution in dimethyl sulfoxide) at 30°C. The reaction was started by adding 10 μl enzyme solution, and the kinetics of hydrolysis was determined by measuring absorption at 410 nm every 10 s for 2 min. Specific activity (units mg^{-1} protein) was calculated and defined as the amount of enzyme releasing 1 μmol 4-nitrophenol min^{-1} (ε, 15.2×10^3 M^{-1} cm^{-1} liter).

Standard operating procedure for investigations with stir bar sorptive extraction (Twister; Gerstel GmbH) in aqueous medium coupled to desorption GC-MS. (i) Sample preparation (6). The minimal medium in 250-ml flasks was prepared in the following way. First, 250-ml Erlenmeyer flasks were cleaned, filled with double-distilled water, and autoclaved to remove volatile organic compounds. The water was replaced with 100 ml phosphate buffer (per liter, 1 g KH_2PO_4, 3.5 g Na_2HPO_4, pH 7.2), and one polydimethylsiloxane-coated stir bar (10 mm of length coated with a 1-mm layer of polydimethylsiloxane; Twister; Gerstel GmbH) was added to each flask and autoclaved again. The minimal medium was completed by adding SL100, CA1000, and trace elements solution (see minimal medium). For cultivation of the bacteria, 10 mM glucose and 20 μg ml^{-1} kanamycin were added to complete the growth medium. For investigating ethyl acetate production, 2 mM 2-butanone was added and the flasks were inoculated with 100 μl of an overnight culture of the *Pseudomonas* strains grown in the same minimal medium without 2-butanone. The flasks were incubated and extracted at the same time on a shaker at 30°C for 48 h and 100 rpm as described elsewhere (7). After removal from the culture medium sample, the stir bar was dried with a paper towel and then transferred into a glass thermal desorption tube for gas chromatography-mass spectrometry (GC-MS) analysis.

GC-MS. To analyze the Twisters, a TDS 2 thermodesorption system (Gerstel GmbH) was used. The glass thermal desorption tube was inserted into the TDS 2 thermodesorption unit, where the stir bar was heated to desorb the metabolites into a cooled injection system with an included programmed temperature vaporizer (CIS 4 PTV; Gerstel GmbH). For thermal desorption the following temperature program was used: starting from 30°C, it ramped at an interval of 30°C min^{-1} up to 150°C, held for 2 min, then ramped at 60°C min^{-1} up to 260°C, holding there for 6 min; the helium flow rate was 50 ml min^{-1}. The PTV injector was cooled and held at −100°C for the total desorption time and then ramped at 12°C s^{-1} in splitless mode to 280°C and held for 3 min. The desorption was done in splitless desorption mode to transfer all compounds from the stir bar to the GC-MS system (GC Agilent 6890 series and MS Agilent 5973 network mass selective detector). The capillary column VF 5ms CP 8944 was used (approximately 30 m by 0.25 mm [inner diameter], 0.25-μm film thickness) with a helium carrier gas column flow rate of 1.9 ml min^{-1}. The MS transfer line and ion source temperatures were set at 280°C, and the MS was run in selected ion monitoring mode. The ions we recorded indicated an *m/z* 72 for 2-butanone and 61 for ethyl acetate. The GC oven temperature was programmed as follows: 34°C for 5 min, ramped at intervals of 5°C min^{-1} to 150°C, and held for 10 min.

Nucleotide sequence accession number. The DNA sequence for the 15-kb chromosomal fragment of *P. veronii* MEK700 contained in pCro5.5 was deposited in the GenBank database under accession number DQ855566.

RESULTS

Construction of the plasposon mini-Tn*5495* and the plasposon delivery vector pCro2a. Transposon mutagenesis remains one of the most useful genetic tools for targeting bacterial genes. Particularly useful are mini-transposons (mini-Tns) derived from Tn*5* (13) which have the transposase outside the inverted repeats of the transposon. This arrangement allows a stable integration of the mini-Tns into the target replicon without further rounds of transposition. After transposition, the cloning of the mini-Tns with the flanking target sequences is greatly improved by including a conditional replication origin in the mini-Tn. Such transposons have been called plasposons (14). Instead of cloning a fragment into a vector, the target DNA is digested by restriction enzymes, and the fragments are circularized by in vitro ligation and then introduced into a permissible host. For transposon mutagenesis of *P. veronii* MEK700, we constructed a new plasposon called mini-Tn*5495*, starting from pUTminiTn*5*-Km1. This pBR322-derived *E. coli* plasmid contains a kanamycin resistance gene between the inner and outer inverted repeats (IRs) IR-I and IR-O of Tn*5* and the Tn*5* tranposase under the *tac* promoter outside the IRs. For mobilization it carries an RP4 *mob* region. The kanamycin resistance gene was replaced in pUTminiTn*5*-Km-1 with a derivative of plasmid pJOE867. The plasmid pJOE867 (2) is derived from a plasmid copy number mutant of Rts1 (28) and contains a kanamycin resistance gene. The new plasmid was called pCro2a, and the transposon was called mini-Tn*5495*. A restriction map is shown in Fig. 1.

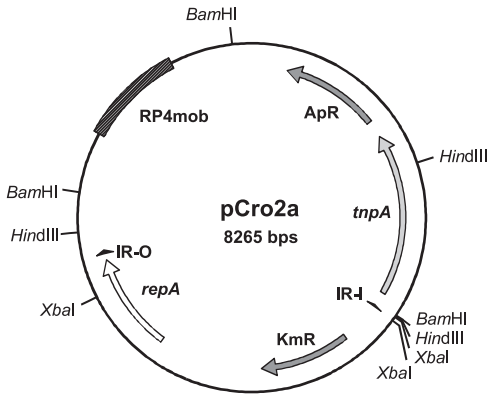


FIG. 1. Restriction map of plasmid pCro2a with mini-Tn*5495*.

Transposon mutagenesis of *P. veronii* with mini-Tn*5495*. For transposon delivery, the plasmid pCro2a was brought into *E. coli* S17-1, which is able to mobilize transfer of pCro2a into *P. veronii* by RP4 integrated in the S17-1 chromosome. Cells from S17-1 and *P. veronii* were mixed and grown together on an LB agar plate overnight at 30°C. Then, the cells were scraped off the plate, and various dilutions were plated on MM agar plates containing glucose as carbon source and nalidixic acid and kanamycin to select transposon mutants of *P. veronii*. On the next day master plates were prepared by transfer of the colonies on new MM agar plates with glucose and the two antibiotics. Finally, the colonies on the master plates were replica plated on various antibiotic-containing MM agar plates, a plate without a carbon source, a plate containing 2-hexanol, a plate with 1-octanol, and as a control, on MM agar plates with glucose. The agar plates without carbon source were brought into an exsiccator, and 2-butanone was supplied in an open glass beaker. Altogether, about 3,000 transposon mutants were tested, and 12 colonies were obtained which showed reduced or no growth at all on 2-butanone and 2-hexanol, whereas growth on 1-octanol or glucose was not affected. From two of the clones which did not show any growth on 2-butanone and 2-hexanol, *P. veronii* MEK700-5 and MEK700-6, chromosomal DNA was isolated and used to clone the transposon target sites.

Analysis of insertion sites of mini-Tn*5495* in *P. veronii* MEK700-5 and MEK700-6. The chromosomal DNA of MEK700-5 and MEK700-6 was digested with various restriction enzymes, and the DNA fragments were ligated again and used to transform *E. coli* JM109. Plasmids were ob-

TABLE 1. Summary of all genes found in the pCro5.5 sequence, the location of the genes, identity of gene products with entries in the GenBank database, and putative functions

Gene name	Start	End	Size (aa)	Highest identity	GenBank accession no.	% Identity (overlaps)[a]	Putative function
gls	Outside	1238	410	*P. syringae* pv. *tomato* strain DC3000	gi 28870298	78 (314/401)	Glycogen synthase
pes	1235	3673	812	*Polaromonas* sp. JS666	gi 67847668	62 (503/804)	Phosphoenolpyruvate synthase
mekA	3843	5492	549	*Rhodococcus* sp. DK17	gi 40787279	61 (325/530)	Monooxygenase
mekB	5576	6625	349	*Rubrivivax gelatinosus* PM1	gi 47571471	66 (230/345)	Homoserine acetyltransferase
mekR	6840	7730	296	*Acinetobacter* sp. NCIMB9871	gi 6277324	35 (103/294)	XylS transcriptional activator
atpD	8098	9474	458	*Methanosarcina acetivorans* C2A	gi 19916394	80 (364/452)	ATP-synthase β-subunit
atpC	9532	9936	134	*Methanosarcina barkeri*	gi 19916393	58 (76/130)	ATP-synthase ϵ-subunit
atp-1	9933	10283	116	*Methanosarcina barkeri*	gi 2605821	55 (50/90)	ATP-synthase gene1
atpB	10590	11291	233	*Methanosarcina acetivorans* C2A	gi 19916390	68 (153/223)	ATP-synthase A-subunit
atpE	11332	11619	95	*Rhodoferax ferrireducens* DSM15236	gi 72603114	86 (58/67)	ATP-synthase C-subunit
atpF	11627	12406	259	*Rhodoferax ferrireducens* DSM15236	gi 72603115	50 (126/249)	ATP-synthase B-subunit
atpA	12401	13948	515	*Methanosarcina acetivorans* C2A	gi 19916387	71 (358/499)	ATP-synthase α-subunit
atpG	13950	14863	304	*Methanosarcina acetivorans* C2A	gi 19916386	57 (173/299)	ATP-synthase γ-subunit

[a] Number of identical amino acids per total number of amino acids which were compared by the BlastX program.

tained from MEK700-5 with EcoRI, AatII, BsrGI, SalI, and XhoI and from MEK700-6 with SalI and XhoI. The largest plasmid from MEK700-5 was pCro5.5, generated with BsrGI. It contained about 15 kb of chromosomal DNA. The largest plasmid from MEK700-6 was obtained with XhoI (pCro6.6) and contained 4.6 kb of chromosomal DNA. Two oligonucleotides, s4037 and s4052, binding inside of mini-Tn*5495* near the ends, were used to sequence the regions flanking the transposon target sites. The DNA analysis for MEK700-5 showed that the transposon was inserted in a gene with similarity to cyclohexanone monooxygenases, and in MEK700-6 it disrupted a gene encoding a putative homoserine acetyltransferase. In addition, upstream of the homoserine acetyltransferase-like gene similarities to cyclohexanone monooxygenase genes were detected. The two sequences obtained from pCro5.5 and pCro6.6 overlapped at the 3′ ends and proved that the two transposon insertions were only 904 bp apart in the *P. veronii* chromosome. This was a clear indication that the transposons inactivated genes involved in 2-butanone degradation. Therefore, the complete 15-kb chromosomal region of pCro5.5 was sequenced.

DNA sequence analysis of pCro5.5. The chromosomal fragment obtained in pCro6.6 was completely included in pCro5.5. Therefore, only pCro5.5 was analyzed in detail. The plasmid was digested with various restriction enzymes and the fragments inserted into pIC20H (15) for DNA sequencing using universal and reverse primers. The gaps still present after this procedure were closed with newly designed primers. Altogether, a region of 15,125 bp was sequenced. The sequences were aligned and analyzed with the DNAstar and BLAST programs. The first identified open reading frame (ORF) is incomplete at the 5′ end. The putative gene was called *gls* according to the deduced protein which showed high similarity to glycogen synthases. The second ORF, *pes*, had a high similarity with genes encoding phosphoenolpyruvate synthase. The next ORF, now called *mekA*, was the one which was hit by mini-Tn*5495* in MEK700-5, and the deduced protein showed a high similarity to BMVO-type enzymes. The next ORF, *mekB*, was the second target of mini-Tn*5495*. The encoded protein had the highest similarity to homoserine acetyltransferases (HAT), which in some organisms is involved in methionine biosynthesis. At a distance of 214 bp to *mekB*, a further ORF was identified which later was called *mekR*. The deduced protein had high similarities to the transcriptional regulatory proteins of the AraC/XylS family. Next to *mekR*, eight ORFs were found which obviously encoded the various subunits of an ATP synthase. The results of this analysis are summarized in Table 1, and the genetic map and locations of the transposon insertions of pCro5.5 and pCro6.6 are displayed in Fig. 2.

Transfer of *mek* genes of *P. veronii* MEK700 to *P. putida* KT2440. According to the DNA sequence analysis of pCro5.5, there are only two or three genes on the cloned fragment involved in 2-butanone and 2-hexanol degradation. These are for sure the *mekA* gene, encoding a BVMO, and the *mekB* gene, encoding the HAT-like enzyme with ester-hydrolyzing properties. To verify the plausible involvement of the transcriptional activator gene *mekR*, genes *mekA* and *mekB* alone

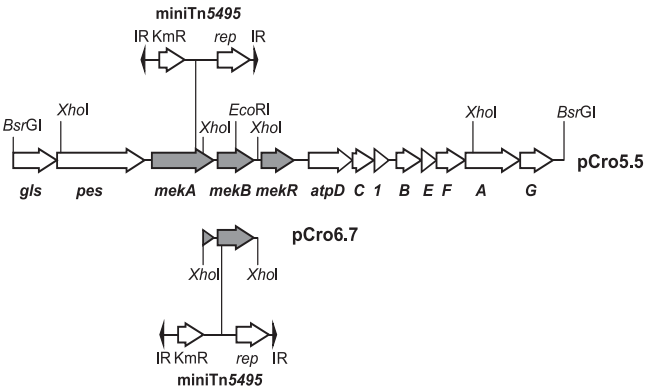


FIG. 2. Restriction map of plasmids pCro5.5 and pCro6.6 with all genes present in mini-Tn*5495* and the ones identified in the cloned *P. veronii* DNA. The insertion sites and orientation of mini-Tn*5495* in pCro5.5 and pCro6.6 are indicated.

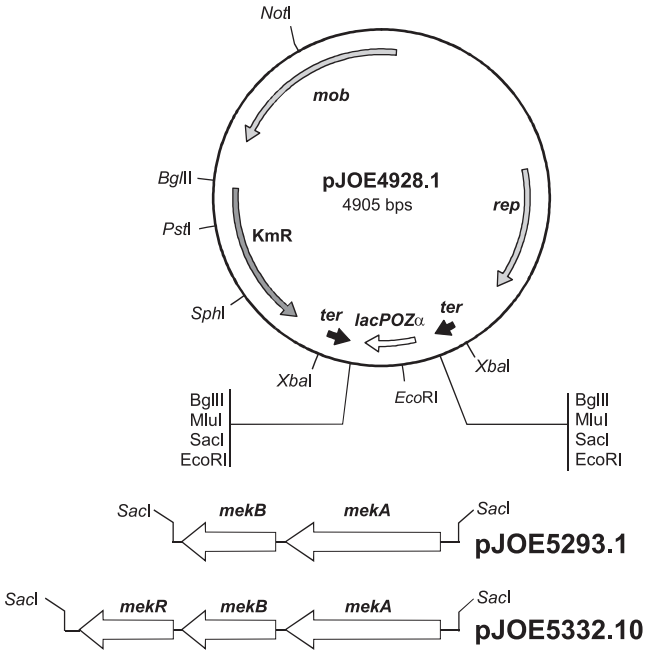


FIG. 3. Restriction map of the positive selection and broad-host-range vector pJOE4928.1 with the kanamycin resistance gene Km1, the *mob* sequence for transfer mobilization, *rep* for replication, the inverted repeated transcriptional terminator (*ter*), and polylinker sequences and the *lacPOZ*α replacement fragment. Below, the PCR-amplified fragments with *mekAB* and *mekABR* are shown, which were inserted into pJOE4928.1 by using endoR SacI to give pJOE5293.1 and pJOE5332.10, respectively.

and together with *mekR* were cotransferred to *P. putida* KT2440, which normally utilizes the putative 2-butanone degradation intermediates ethanol and acetate but not 2-butanone itself. For this purpose a new broad-host-range, positive selection vector, pJOE4928.1, was constructed. The vector is based on the *Bordetella pertussis*-derived plasmid pBBR1MCS2 (with kanamycin resistance). The *lacZ*α fragment was deleted and replaced by two long inverted repeats consisting of a transcription terminator and polylinker sequences. Between the inverted repeats the *lacZ*α fragment with *lac* promoter and operator (*lacPOZ*α) was inserted. The positive selection is based on the fact that bacteria are usually not able to replicate long inverted repeats, except the repeats are separated by a unique sequence of at least 50 bp (24). The restriction map of pJOE4928.1 is shown in Fig. 3. The genes *mekA* and *mekB* were amplified by long-template PCR together with the *mekA* upstream region till the 3′ end of the *pes* gene. In addition, in a second PCR *mekAB* and *mekR* were amplified. With the SacI restriction sites in the primers, the two fragments were inserted into pJOE4928.1, replacing the *lacPOZ*α fragment to give pJOE5293.1 and pJOE5332.10. The amplified fragments were completely sequenced to exclude mutations by PCR, then *E. coli S17*.1 was transformed with the two plasmids and mated with *P. putida* KT2440, and transconjugants were selected on LB agar plates with nalidixic acid and kanamycin. The colonies obtained in this way were streaked on MM agar plates without carbon source and incubated in a sealed exsiccator with 2-butanone provided separately as described before. Only *P. putida* with pJOE5332.10 was able to grow on 2-butanone. This clearly demonstrates that all three genes are, first, necessary and second, sufficient to allow growth on 2-butanone, in case a strain can productively metabolize putative 2-butanone (MEK) degradation intermediates like ethanol and acetate.

Expression of *mekA* in *E. coli*. The most likely function of the *mekA*-encoded enzyme is the oxidation of 2-butanone to ethyl acetate. The gene was amplified from *P. veronii* DNA by PCR and inserted into the L-rhamnose-inducible *E. coli* expression vector pWA21 to give pJOE5302.3. The plasmid was brought into *E. coli* JM109, and the strain was induced in LB liquid medium for 6 h at 30°C. An SDS-PAGE analysis of the *E. coli* crude protein extract separated into soluble and insoluble protein fractions showed a strong inducible protein band in the insoluble protein fraction exhibiting the expected size for MekA (data not shown).

This indicates that most protein is synthesized in an inactive form of inclusion bodies, disqualifying the recombinant *E. coli* strain for further investigation of the *mekA*-encoded BVMO.

Ethyl acetate production by *mekB* mutant *P. veronii* MEK700-6. Due to inclusion body formation, it was not possible to study the Baeyer-Villiger reaction catalyzed by the *mekA* gene product in the recombinant *E. coli* strain. Therefore, the mutant *P. veronii* MEK700-6 with the transposon inserted into *mekB* was investigated for ethyl acetate production. With an overnight culture of this strain in MM glucose medium, a fresh MM with glucose and 2 mM 2-butanone was inoculated and incubated for 48 h at 30°C. The hydrophobic substrate and the reaction products were absorbed to Twisters (coated stir bars; stir bar sorptive extraction method developed by Baltussen et al. [7]) and analyzed by gas chromatography. Half of the 2-butanone added (2 mM) was converted into ethyl acetate (the recovery rate of substrate not transformed and product formed was 90%). No ethyl acetate production was found in the control strain *P. veronii* MEK700-5, which has the transposon inserted into the BMVO gene *mekA*, but here a small amount of the 2-butanone was reduced to 2-butanol (presumably unspecifically) (data not shown). The experiments hereby proved that MekA indeed is a Baeyer-Villiger monooxygenase.

The *mekB* gene encodes a highly active esterase-like enzyme. Ethyl acetate produced by MekA is either simply hydrolyzed to ethanol and acetate in an esterase-like reaction or via the transfer of the acetyl moiety to coenzyme A (CoA), giving acetyl-CoA and ethanol, as suggested by the sequence relationship of MekB with HAT. In the latter case, this would save the activation step of acetate via ATP. To characterize MekB biochemically, the gene was amplified by PCR without start codon. A BamHI site upstream of the second codon and a HindIII site behind the stop codon in the primers were used to insert the PCR fragment into the L-rhamnose-inducible expression vector pWA21. Hereby, the gene was fused in frame to the first five codons of *lacZ* and six-histidine codons present in the vector. The plasmid called pJOE5358.1 was used to transform JM109. An ampicillin-resistant transformant was grown in LB broth, induced with L-rhamnose, and tested for esterase-like activity by incubating crude cell extract with 4-nitrophenyl acetate. The rapid appearance of a yellow color due to hydrolysis of 4-nitrophenyl acetate to nitrophenol indicated the presence of an esterase activity. Therefore, the strain was grown in

TABLE 2. Purification of MekB from JM109pJOE5358.1 by IMAC[a]

Purification step	Total protein (mg)	Total activity (U mg^{-1})	Sp act (U mg^{-1})	Purification (fold)	Recovery (%)
Crude extract	21	504	24	1.0	100
Ni-NTA resin	1.94	421	217	9.0	83

[a] Enzyme activity was measured with 4-nitrophenyl acetate.

a larger volume and induced by L-rhamnose, and the MekB protein was isolated by IMAC. The results of the purification are summarized in Table 2. The specific activity of MekB in the crude extract was 24 U mg^{-1} protein and, after purification, 217 U mg^{-1} protein. The protein was more than 85% pure according to SDS-PAGE (Fig. 4). The activity of the enzyme decreased with increasing chain length of the acid moiety of the substrate. With 4-nitrophenyl butyrate, a specific activity of only 3.4 U mg^{-1} protein was found. The activity with 4-nitrophenyl formiate could not be measured due to the fast spontaneous hydrolysis of this compound. To find out more about the substrate specificity of MekB, esterase activities were determined in pH stat assays with 100 mM substrate at 30°C. The esters are listed in Table 3 together with the specific activities obtained under these conditions. The highest activities again were obtained with acetic acid esters, but also the alcohol group obviously plays an important role, as compounds with two carbon atoms in the alcohol moiety, i.e., vinyl and ethyl acetate, are by far the preferred substrates.

DISCUSSION

Transfer of only three genes from *P. veronii* to *P. putida* KT2440 can confer to this strain the capability to degrade 2-butanone. These are, first, a gene for a BVMO, second, a gene exhibiting a high homology with HAT, and third, a transcriptional regulator gene of *araC-xylS*. In some bacteria which are able to grow on alkanes and cyclic alkanes, BVMO and esterase genes may be part of a larger alkane-oxidizing gene cluster, comprising alkane hydroxylase genes as well as alcohol and aldehyde dehydrogenase genes (29). This is not the case in *P. veronii* MEK700. Here the three essential genes are flanked by housekeeping genes, a phosphoenolpyruvate synthase and glycogen synthase gene for gluconeogenesis and carbohydrate storage on one side and an ATP synthase on the other side, which obviously have nothing to do with 2-butanone degradation. In a *Brachymonas* species capable of growing on cyclohexane, two gene clusters were identified: one was for the hydroxylation of cyclohexane, whereas the second one contained all genes for degradation of cyclohexanol to adipic acid (10). The presence of a second gene cluster for hydroxylation of short chain alkanes in *P. veronii* seems to be unlikely, since this strain does not grow on hexane or cyclohexane (data not shown; butane or pentane have not been tested). In addition, the genes are induced by growth with methyl ketones and not by the corresponding alkanes, as seen in mycobacteria. On the other hand, the strain grows on 2-hexanol, which in addition needs an alcohol dehydrogenase. This gene is not part of the gene cluster. It might be that the secondary alcohol is oxidized by an unspecific alcohol dehydrogenase. Maybe it represents the same activity, which was also observed in the mutant *P. veronii* MEK700-5, where some 2-butanone was reduced to the secondary alcohol derivative.

The *mek* genes of *P. veronii* seem to be specific for short chain methyl ketones like 2-butanone and 2-hexanone. This is assumed from the substrate specificity of the *mekB*-encoded esterase, which prefers esters with short chain acids and alcohols. The investigation of the BVMO concerning substrate specificity was hampered by the inclusion body formation of the heterologously expressed gene *mekA*. Nevertheless, since *P. veronii* does not grow on cyclohexanone, one can conclude that the monooxygenase prefers linear short chain methyl ketones. MekA is a typical Baeyer-Villiger monooxygenase as seen from the presence of a BVMO-identifying motif, FXGX XXHXXXW (20), which is found in MekA at amino acid position 170 to 180 and from a phylogram. In the meantime, there are hundreds of protein sequences with high similarity to BVMOs deposited in databases, mainly from the sequencing projects of bacterial chromosomes, but the functions of most of these sequences are putative. Therefore, MekA was aligned with a group of BVMOs, the genes of which have been actually cloned, heterologously expressed, and for which the corresponding enzyme activity has been measured. This group comprises cyclohexanone monooxygenases from *Acinetobacter* sp. strain SE19 and strain NCIMB 9871 (12), *Brachymonas petroleovorans* (10), *Arthrobacter* sp. BP2 and *Rhodococcus* sp. strains Phi1 and Phi2 (11), *Xanthobacter flavus* ZL5 (45), and from *Brevibacterium* sp. strain HCU (9), the cyclopentanone monooxygenase of *Comamonas testosteroni* NCIMB 9872 (25), cyclododecanone monooxygenase from *Rhodococcus ruber* SC1 (32), a cyclopentadecanone monooxygenase from *Pseudomonas* sp. strain HI-70 (26), a 4-hydroxyacetophenone monooxygenase from *P. putida* ACB (27), a phenylacetone monooxygenase from *Thermobifida fusca* (21), a

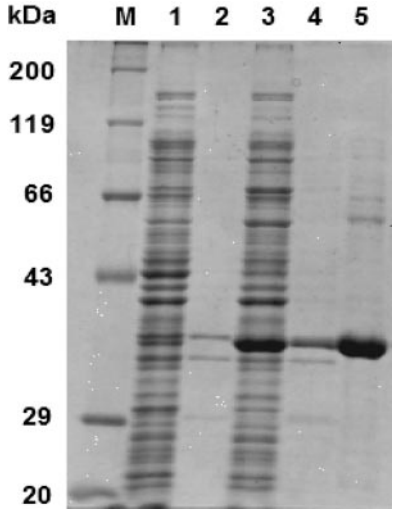
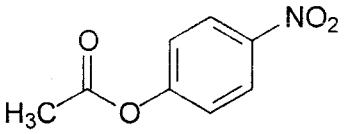
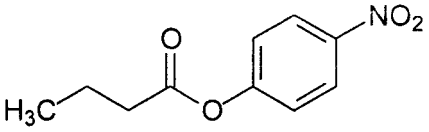
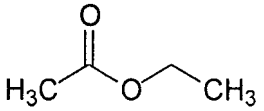
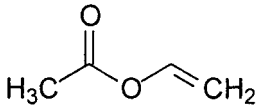
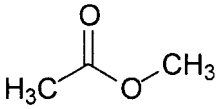
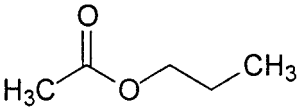
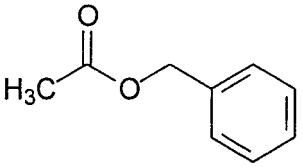
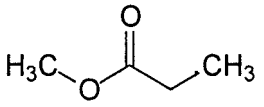
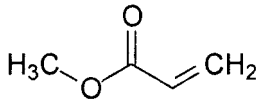
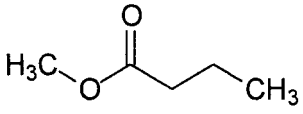
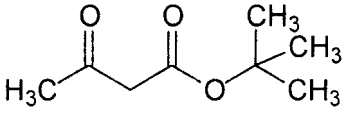


FIG. 4. SDS-PAGE of crude extracts and purified enzyme from *E. coli* JM109 with plasmid pJOE5358.1 containing the *mekB* gene under control of the L-rhamnose-inducible promoter. Lanes: 1, crude extract from noninduced cells, soluble fraction; 2, crude extract from noninduced cells, insoluble fraction; 3, crude extract from L-rhamnose-induced cells, soluble fraction; 4, crude extract from L-rhamnose-induced cells, unsoluble fraction; 5, MekB purified by IMAC.

TABLE 3. Specific activity of purified MekB with various esters as substrate

Substrate	Chemical structure	Sp act (U mg^{-1} protein)
4-Nitrophenyl acetate		217
4-Nitrophenyl butyrate		3.4
Ethyl acetate		230
Vinyl acetate		288
Methyl acetate		78
Propyl acetate		77
Benzyl acetate		30
Methyl propionate		28
Methyl acrylate		22
Methyl butyrate		2.8
tert-Butyl acetoacetate		1.4

steroid monooxygenase from *Rhodococcus rhodochrous* (38), and an alkane monooxygenase of *P. fluorescens* DSM 50106 (31). In addition to these characterized BVMOs, the putative cyclohexanone monooxygenase from *Rhodococcus* sp. strain DK17 (accession number AAR90196) was added to this group since this enzyme, according to the BlastP analysis, shares the highest identity in amino acid sequence with the *mekA* gene product. Of all these enzymes, only the BVMO from *P. fluorescens* seems to be specific for short aliphatic methyl ketones. This is not reflected in the phylogram, in which the BVMO from *P. veronii* has the strongest homology with the putative BVMO of *Rhodococus* sp. strain DK17, as expected, and the cyclopentanone monooxygenase of *C. testosteroni* (Fig. 5). No pattern can be recognized in the tree concerning the bacterial origin of the genes or the substrate

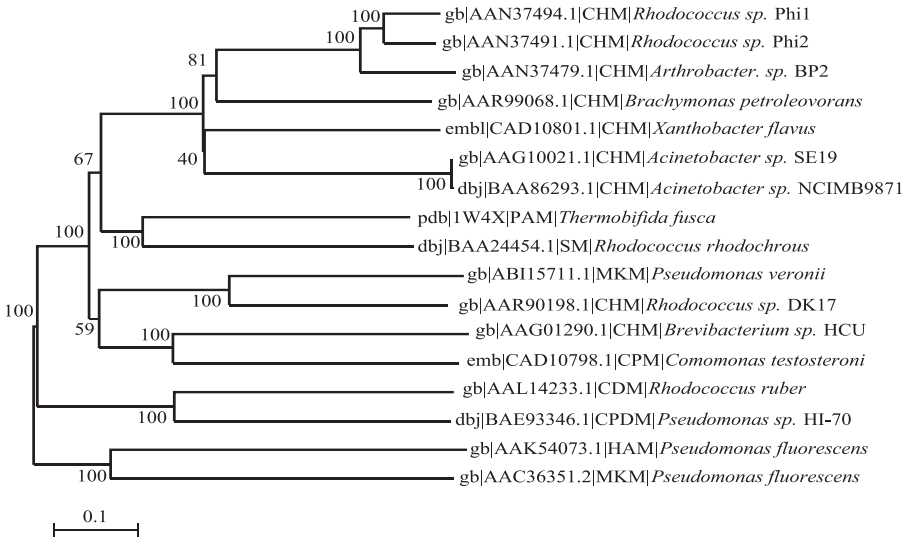


FIG. 5. Phylogram of Baeyer-Villiger monooxygenase sequences. The unrooted tree was obtained by multiple alignments of the various amino acid sequences with the ClustalX (version 1.8) program, and the tree was calculated by the neighbor-joining method and visualized by the njplot program in this program package. Abbreviations: CHM, cyclohexanone monooxygenase; MKM, methyl ketone monooxygenase; CPM, cyclopentanone monooxygenase; CDM, cyclodecanone monooxygenase; CPDM, cyclopentadecanone monooxygenase; HAM, 4-hydroxyacetophenone monooxygenase; PAM, phenylacetone monooxygenase; SM, steroid monooxygenase.

specificity. Only the cyclohexanone monooxygenases seem to form a subgroup (except for a cyclohexanone monooxygenases of *Brevibacterium* sp. strain HCU).

Concerning the biochemistry of degradation of ketonic substrates in general, the intermediate nature of an ester has been for the first time proven unequivocally by identification of ethyl acetate during 2-butanone turnover in the *mekB* mutant *P. veronii* MEK700-6.

The most surprising finding was, however, that the gene *mekB* encoding the esterase activity had similarity with homoserine acetyltransferases and not with esterases as expected. On the other hand, if the situation is similar to some glycosidases, there might be just one mutation necessary to convert the transferase activity into a hydrolysis activity. This was observed for a β-glycosidase of *Thermus thermophilus*, where several single mutations changed the main activity from hydrolysis action to transglycosylation and vice versa (18). Homoserine acetyltransferases play a role in de novo methionine biosynthesis of many bacteria. For example, in *Bacillus subtilis* the homoserine acetyltransferase transfers acetate from acetyl-CoA to homoserine forming *O*-acetyl homoserine (39). In other bacteria, like *E. coli*, a homologous enzyme transfers succinate to homoserine to give succinylhomoserine. From both intermediates, homocysteine is synthesized via transsulfuration with cysteine or directly with sulfhydrolase and sulfide (23). Due to the similarity of MekB and homoserine acetyltransferase, it was tempting to speculate that MekB is able to transfer the acetyl moiety from ethyl acetate to CoA instead of just directly hydrolyzing the ester. The latter would need an otherwise unnecessary activation step, i.e., use of ATP in phosphorylating the acetate liberated to produce acetylphosphate followed by the transfer of the acetyl group to CoA. Nevertheless, this energy-saving mechanism seems unlikely, based on the very high rate of ester hydrolysis exhibited by the *mekB*-derived presumptive transferase. Therefore, it is reasonable to assume that methyl ketones are degraded in *P. veronii* MEK700 in the following way: the methyl ketones penetrate the cytoplasm membrane, maybe assisted by an unspecific transporter, and first bind to the transcriptional activator MekR, which induces transcription of *mekA* and *mekB*. Then, the newly produced MekA oxidizes the methyl ketone to the corresponding ester, which is hydrolyzed by MekB to alcohol and acetate, which are further metabolized by housekeeping genes.

The same or similar degradation pathways might occur in other bacteria. For example, in the *Rubrivivax gelatinosus* PM1 chromosome (accession number N2_AAEM001000018) and on the *Rhodococcus* sp. strain RHA1 plasmid pRHL1 (accession number NC_008269), a *mekB* homologous gene is in the immediate vicinity of a putative BVMO gene.

The knowledge about the degradation of butanone should be helpful in improving the performance of biofiltration systems in biological waste air purification. It should allow follow up of the genes of butanone degradation in the filter community and to understand their regulation. The latter is important, as in technical systems frequently mixtures of compounds are used in which some substances may inhibit butanone degradation by repression. Secondly, the easy transfer of the *mek* genes to other *Pseudomonas* strains will be useful to generate strains with multiple pathways, thereby increasing the potentials and the stability of the degradative processes in technical waste air-cleaning plants.

ACKNOWLEDGMENTS

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