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Mesaconase Activity of Class I Fumarase Contributes to Mesaconate Utilization by *Burkholderia xenovorans*

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Pseudomonas aeruginosa*, *Yersinia pestis*, and many other bacteria are able to utilize the C_5-dicarboxylic acid itaconate (methylenesuccinate). Itaconate degradation starts with its activation to itaconyl coenzyme A (itaconyl-CoA), which is further hydrated to (*S*)-citramalyl-CoA, and citramalyl-CoA is finally cleaved into acetyl-CoA and pyruvate. The xenobiotic-degrading betaproteobacterium *Burkholderia xenovorans* possesses a *P. aeruginosa*-like itaconate degradation gene cluster and is able to grow on itaconate and its isomer mesaconate (methylfumarate). Although itaconate degradation proceeds in *B. xenovorans* in the same way as in *P. aeruginosa*, the pathway of mesaconate utilization is not known. Here, we show that mesaconate is metabolized through its hydration to (*S*)-citramalate. The latter compound is then metabolized to acetyl-CoA and pyruvate with the participation of two enzymes of the itaconate degradation pathway, a promiscuous itaconate-CoA transferase able to activate (*S*)-citramalate in addition to itaconate and (*S*)-citramalyl-CoA lyase. The first reaction of the pathway, the mesaconate hydratase (mesaconase) reaction, is catalyzed by a class I fumarase. As this enzyme (Bxe_A3136) has similar efficiencies (k_{cat}/K_m) for both fumarate and mesaconate hydration, we conclude that *B. xenovorans* class I fumarase is in fact a promiscuous fumarase/mesaconase. This promiscuity is physiologically relevant, as it allows the growth of this bacterium on mesaconate as a sole carbon and energy source.

Itaconate (methylenesuccinate) is an industrially important fungal product and a common carbon source for various soil bacteria (1). Still, the importance of other C_5-dicarboxylic acids such as ethylmalonate, methylsuccinate, citramalate (α-methylmalate), and mesaconate (methylfumarate) was neglected for decades. Yet, the participation of these compounds in several central metabolic pathways has been shown in the last 15 years. Examples are the autotrophic 3-hydroxypropionate bi-cycle as well as the anaplerotic ethylmalonyl coenzyme A (ethylmalonyl-CoA) pathway and methylaspartate cycle functioning for the assimilation of C_2 units (2–6). Furthermore, mesaconate and citramalate are intermediates of the methylaspartate pathway of glutamate fermentation functioning in many (facultative) anaerobic bacteria (7).

Itaconate was recently identified as a mammalian metabolite whose production is substantially induced during macrophage activation (8, 9). As itaconate is known as a potent inhibitor of the glyoxylate cycle, a metabolic pathway important for many pathogens during infection, itaconate production by macrophages is regarded as part of the antibacterial response of macrophages (9–11). Interestingly, many pathogens contain genes involved in itaconate assimilation (11). The capability to degrade itaconate promotes the survival and infectivity of pathogens inside the hosts (10–12). Bacterial itaconate degradation involves three steps, i.e., itaconate activation to itaconyl-CoA, its hydration (via mesaconyl-CoA) to (*S*)-citramalyl-CoA, and the cleavage of (*S*)-citramalyl-CoA into acetyl-CoA and pyruvate (Fig. 1) (11, 13). Itaconate degradation was studied for two pathogens, *Yersinia pestis* and *Pseudomonas aeruginosa*, and the genes involved in this process were identified (11). The metabolic pathways are similar in the two species. However, they use different sets of mostly unrelated enzymes to convert itaconate into acetyl-CoA and pyruvate. While the itaconate degradation operon in *Y. pestis* consists of only three genes necessary for the utilization of itaconate, the *P. aeruginosa* itaconate degradation cluster possesses three additional genes. The gene products of those three genes were hypothesized to be involved in the channeling of other C_5-dicarboxylic acids into the itaconate degradation pathway (Fig. 2). *P. aeruginosa* is able to grow on mesaconate (14), and one of these genes, *pa0881*, encoding an MmgE-PrpD family protein, was proposed to function as mesaconate hydratase (mesaconase), converting mesaconate into (*S*)-citramalate (11).

The nonpathogenic xenobiotic-degrading soil betaproteobacterium *Burkholderia xenovorans* possesses a gene cluster similar to the *P. aeruginosa* itaconate degradation cluster (11, 15) (Fig. 2). This versatile bacterium bears one of the largest bacterial genomes and has great potential for environmental biotechnological applications, thus being an interesting model organism for studying microbial metabolism. The *B. xenovorans* itaconate degradation cluster also contains a gene, *bxe_b2582*, encoding an MmgE-PrpD family protein with 63% sequence identity to the proposed mesaconase PA0881. In this study, we show that *B. xenovorans* is able to grow on both itaconate and mesaconate and that mesaconate is degraded through conversion to (*S*)-citramalate, activation to (*S*)-citramalyl-CoA, and (*S*)-citramalyl-CoA cleavage into pyruvate and acetyl-CoA. In order to identify and characterize mesaconase

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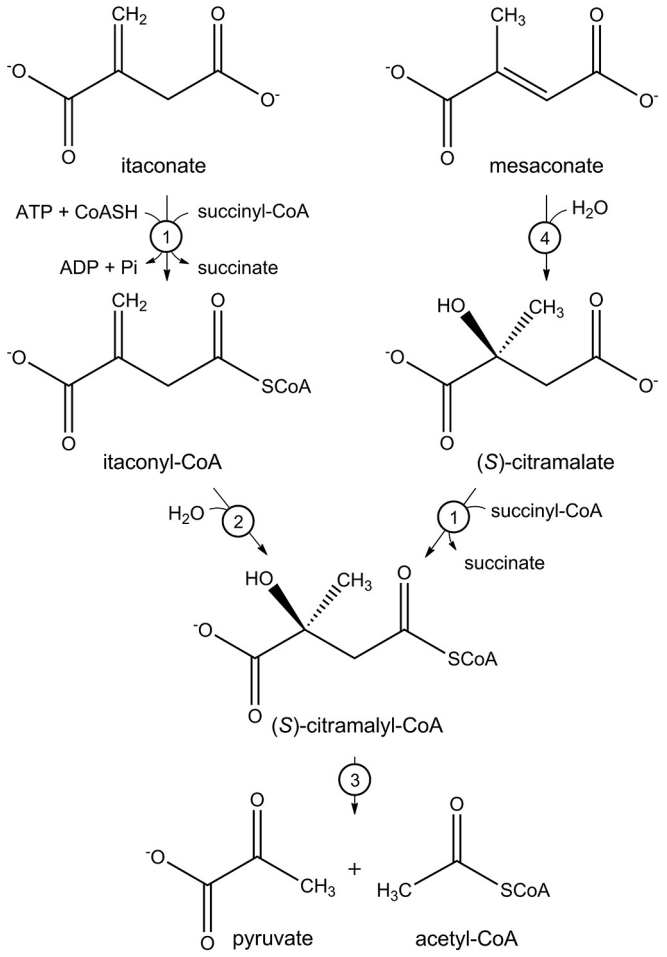

FIG 1 Itaconate and mesaconate metabolism in *Pseudomonas aeruginosa* and *Burkholderia xenovorans* (adapted from reference 11). Enzymes: 1, succinyl-CoA:itaconate-CoA transferase (referred to in the text as itaconate-CoA transferase); 2, itaconyl-CoA isomerase/mesaconyl-CoA hydratase (referred to in the text as itaconyl-CoA hydratase); 3, (*S*)-citramalyl-CoA lyase; 4, mesaconase. Note that itaconate-CoA transferase is able to catalyze both itaconate and (*S*)-citramalate activation (11) and that succinyl-CoA synthetase is capable of activating itaconate as well.

in this bacterium, we cloned and heterologously expressed *bxe_b2582* in *Escherichia coli*. However, although the produced protein was soluble, it did not catalyze the proposed mesaconase reaction, leaving its function unknown. Instead, an iron-dependent fumarase (class I fumarase) of *B. xenovorans* that is not encoded by the itaconate degradation cluster is responsible for the hydration of mesaconate during mesaconate utilization in *B. xenovorans* (Fig. 1). This study shows how enzyme promiscuity allows bacteria to access novel substrates that otherwise would remain untouched.

MATERIALS AND METHODS

Materials. Chemicals were obtained from Fluka (Neu-Ulm, Germany), Sigma-Aldrich (Deisenhofen, Germany), Merck (Darmstadt, Germany), Serva (Heidelberg, Germany), or Roth (Karlsruhe, Germany). Biochemicals were from Roche Diagnostics (Mannheim, Germany), AppliChem (Darmstadt, Germany), or Gerbu (Craiberg, Germany). Materials for cloning and expression were purchased from MBI Fermentas (St. Leon-Rot, Germany), New England BioLabs (Frankfurt, Germany), Novagen

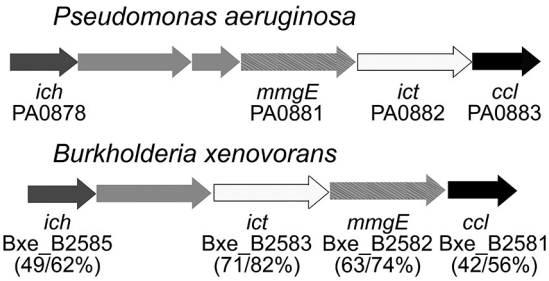

FIG 2 Gene clusters encoding enzymes of itaconate degradation in *P. aeruginosa* and *B. xenovorans* (15, 47). The cluster contains genes encoding hypothetical proteins (three in *P. aeruginosa*, two in *B. xenovorans*) as well as the following genes: *ccl*, for (*S*)-citramalyl-CoA lyase; *ich*, for itaconyl-CoA hydratase; *ict*, for succinyl-CoA:itaconate-CoA transferase. The percentage of sequence identity/similarity of the corresponding proteins to those from *P. aeruginosa* is shown in parentheses.

(Schwalbach, Germany), Genaxxon Bioscience GmbH (Biberach, Germany), Biomers (Ulm, Germany), or Qiagen (Hilden, Germany). Materials and equipment for protein purification were obtained from GE Healthcare (Freiburg, Germany), Macherey-Nagel (Düren, Germany), Pall Corporation (Dreieich, Germany), or Millipore (Eschborn, Germany).

Syntheses. Acetyl-CoA, succinyl-CoA, and itaconyl-CoA were synthesized from their anhydrides by the method of Simon and Shemin (16). (*S*)-Citramalyl-CoA was synthesized enzymatically with recombinant (*S*)-malyl-CoA/β-methylmalyl-CoA/(*S*)-citramalyl-CoA lyase from *Chloroflexus aurantiacus* (3), as described previously (11).

Microbial strain and growth conditions. *Burkholderia xenovorans* strain LB400 (DSMZ 17367) was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). The cells were grown under aerobic conditions at 30°C with acetate, mesaconate, or itaconate (20 mM) in minimal medium containing NH_4Cl (1.2 g liter^{-1}), $MgSO_4 \cdot 7H_2O$ (0.4 g liter^{-1}), and $CaCl_2 \cdot 2H_2O$ (0.1 g liter^{-1}). After autoclaving, 10 ml trace element mixture (EDTA, 500 mg; $FeSO_4 \cdot 7H_2O$, 300 mg; $MnCl_2 \cdot 4H_2O$, 3 mg; $CoCl_2 \cdot 6H_2O$, 5 mg; $CuCl_2 \cdot 2H_2O$, 1 mg; $NiCl_2 \cdot 6H_2O$, 2 mg; $Na_2MoO_2 \cdot 2H_2O$, 3 mg; $ZnSO_4 \cdot 7H_2O$, 5 mg; H_3BO_3, 2 mg; distilled water, 1 liter), 8 ml vitamin solution (niacin, 200 mg; niacinamide, 200 mg; thiamine hydrochloride, 400 mg; biotin, 8 mg; distilled water, 1 liter), and 20 ml of 1 M phosphate buffer (prepared from 1 M K_2HPO_4 solution by adjusting pH value to 7.0 with 1 M NaH_2PO_4) were added aseptically per liter of medium. Vitamins and trace element mixture were filter sterilized; all other solutions were autoclaved. The pH was adjusted to 7.0 using NaOH.

Preparation of cell extracts. Cell extracts were prepared under oxic conditions using a mixer-mill (MM200; Retsch, Haare, Germany). Cells (100 to 150 mg) were suspended in 0.5 ml of 20 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS)–KOH, pH 6.9, 0.1 mg ml^{-1} DNase I, and 0.5 mM dithiothreitol (DTT) in 2.2-ml Eppendorf vials. Then, 1 g of glass beads (diameter, 0.1 to 0.25 mm) was added to the suspension, and the cells were treated in the mixer-mill for 10 min at 30 Hz. This was followed by a centrifugation step (14,000 × *g*, 4°C, 10 min), and the supernatant (cell extract) was used for enzyme assays. The protein content of the cell extract was 6 to 20 mg ml^{-1}.

Enzyme assays. Spectrophotometric enzyme assays (0.5-ml assay mixture) were performed aerobically in 0.5-ml cuvettes at 30°C, unless otherwise indicated. Anaerobic assays were done in an anaerobic chamber. One enzyme unit (U) corresponds to 1 μmol substrate converted per minute.

The activity of itaconate conversion into acetyl-CoA and pyruvate was measured spectrophotometrically as itaconate-dependent pyruvate formation with phenylhydrazine at 324 nm ($\varepsilon_{\text{pyruvate phenylhydrazine}} = 11.52$ mM^{-1} cm^{-1}) (2). The assay mixture contained 100 mM MOPS-KOH

(pH 6.9), 5 mM $MgCl_2$, 5 mM DTT, 3.5 mM phenylhydrazine, 0.5 mM succinyl-CoA, 10 mM itaconate, and cell extract. (*S*)-Citramalate, citraconate, and mesaconate conversions were measured similarly to the conversion of itaconate except that itaconate was replaced with (*S*)-citramalate and mesaconate (10 mM) in the reaction mixture. To test free CoA as a possible CoA donor for the conversions, succinyl-CoA was replaced with ATP (3 mM) and CoA (0.5 mM) in the reaction mixture.

Fumarase activity was measured under anoxic conditions in quartz cuvettes spectrophotometrically at 240 nm ($\varepsilon_{fumarate}$ = 2.4 mM^{-1} cm^{-1} [pH 7.5] [17], constant at pH 6.9 to 8.5 [data not shown]). The assay mixture contained 100 mM MOPS-KOH (pH 6.9), 5 mM $MgCl_2$, 5 mM DTT, 0.4 mM fumarate (for forward reaction) or 5 mM (*S*)-malate (for reverse reaction), and cell extract. K_m and V_{max} values were determined by varying the concentration of fumarate from 0.02 to 5 mM and that of malate from 0.2 to 5 mM while keeping the other concentrations constant. For fumarate concentrations above 1 mM, a quartz cuvette with a path length of 1 mm was used. The reaction was started by the addition of cell extract or 0.06 to 0.5 μg of reactivated purified protein. To test substrate specificity, the activity of purified class I fumarase was assayed with (*R*)-malate (20 mM), (*R*)-citramalate (20 mM), maleate (0.4 mM), or citraconate (0.4 mM) as a substrate.

Mesaconase activity was measured under anoxic conditions in quartz cuvettes spectrophotometrically at 250 nm ($\varepsilon_{mesaconate}$ = 2.26 mM^{-1} cm^{-1} [pH 8.0] [18], constant at pH 6.9 to 8.5 [data not shown]). The assay mixture was the same as for the fumarase reaction except that fumarate or (*S*)-malate was replaced with mesaconate or (*S*)-citramalate. K_m and V_{max} values were determined by varying the concentration of mesaconate from 0.02 to 0.7 mM and that of (*S*)-citramalate from 0.3 to 5 mM while keeping the other concentrations constant. The reaction was started by the addition of cell extract or 0.4 to 0.9 μg of the reactivated purified protein.

Succinyl-CoA:itaconate-CoA transferase, itaconyl-CoA hydratase, and (*S*)-citramalyl-CoA lyase activities were measured using an ultraperformance liquid chromatography (UPLC)-based assay described in reference 11. Succinyl-CoA:(*S*)-citramalate-CoA transferase activity was measured as described for succinyl-CoA:itaconate-CoA transferase, except that itaconate was replaced with (*S*)-citramalate (10 mM).

Reactivation of fumarases. The iron-sulfur clusters of class I fumarate hydratase were partially inactivated when exposed to oxygen; therefore, the enzyme needed to be reactivated before performing the enzymatic assays. For reactivation (18), the purified fumarate hydratase dissolved in 50% (vol/vol) glycerol was mixed with 1 volume of reactivation buffer [50 mM Tris-HCl, pH 8.2; 25 mM DTT; 0.25 mM ferrous(II) ammonium sulfate]. The solution was incubated under anoxic conditions at 30°C for 1.5 to 2 h and then kept at 4°C until the enzyme assays were performed. The anaerobization procedure was performed in glass vials with butyl rubber stoppers and consisted of eight cycles of evacuation (1 min) and filling with N_2 (1 min) and venting of the final N_2 overpressure. The activated protein remained active for several days under these conditions.

Analysis of the products of itaconate, (*S*)-citramalate, and mesaconate conversions by cell extracts of *B. xenovorans*. The reaction mixture contained 100 mM MOPS-KOH (pH 6.9), 5 mM $MgCl_2$, 5 mM DTT, 10 mM substrate [itaconate, (*S*)-citramalate, or mesaconate], and cell extract. The reaction was started by the addition of succinyl-CoA (1 mM). After appropriate time intervals, 20 μl of the assay mixture was transferred to ice and the reaction was stopped by addition of 10 μl of 1 M HCl. Protein was removed by centrifugation (20,800 × *g*, 4°C, 20 min), and the samples were analyzed by RP-C_{18} UPLC, as described in reference 11.

Cloning of fumarase genes from *B. xenovorans*. The fumarase genes were amplified by PCR from *B. xenovorans* LB400 chromosomal DNA with the Q5 high-fidelity DNA polymerase (New England BioLabs) according to the manufacturer's instructions using the following primers: FumI_Bxe_fwd (CATATCGAAGGTCGTCATGTGTCCGACAACAAGCGC) and FumI_Bxe_rev (GACAGCTTATCATCGATATCAAACGGTCGCGACCGG) for *bxe_A3136*, encoding a class I enzyme, and FumII_Bxe_fwd (CATATCGAAGGTCGTCATATGACTGAAGACGTACGAATGGAGCGTGAC) and FumII_Bxe_rev (GACAGCTTATCATCGATATCACGCCGCCGGATGCCC) for *bxe_A1038*, encoding a class II enzyme. Primers were constructed with NEB Builder (New England BioLabs) and contained adapter sequences for the cloning vector pET16b (underlined). Thirty-four cycles were conducted in an automated thermocycler under the following conditions: a 20-s (3 min in the first cycle) denaturation at 98°C, annealing for 30 s at 68°C (for *bxe_A3136*) or 72°C (for *bxe_A1038*), and primer extension for 1.5 min (5 min in the final cycle) at 72°C. The PCR products were purified and ligated into the pET16b vector (restricted with HindIII and NdeI), using the Gibson assembly cloning kit (New England BioLabs, Frankfurt, Germany) according to the manufacturer's protocol.

Heterologous expression of fumarases in *Escherichia coli*. Recombinant enzymes were produced in *E. coli lysY* cells (New England BioLabs, Frankfurt, Germany) that had been transformed with the corresponding plasmids. The cells were grown aerobically at 37°C in LB medium supplemented with 100 μg ml^{-1} ampicillin, 100 μM $FeSO_4$, and 100 μM Fe(II) citrate. Expression was induced at an optical density at 578 nm (OD_{578}) of 0.4 to 0.5 with 0.8 mM isopropyl-thio-β-D-galactoside, and the temperature was lowered to 20°C. After additional growth for 12 to 15 h, cells were harvested by centrifugation (4,500 × *g*, 10 min, 4°C) and stored at −20°C until use.

Purification of recombinant enzymes. Frozen *E. coli* cells from the respective heterologous expression of *B. xenovorans* fumarases were suspended in a buffer containing 40 mM Tris-HCl (pH 7.8), 0.1 mg ml^{-1} DNase I, and 0.5 mM DTT (1.5 ml resuspension buffer per 1 g cell mass). The suspensions were passed twice through a chilled French pressure cell at 137 MPa, and the cell lysate was centrifuged for 1 h at 100,000 × *g* and 4°C. The supernatant was loaded onto a 1 ml Protino nickel-nitrilotriacetic acid (Ni-NTA) column (Macherey-Nagel) previously equilibrated with buffer A (50 mM Tris-HCl, pH 7.8, 300 mM NaCl, 5 mM $MgCl_2$). Purification was carried at a flow rate of 1 ml min^{-1}. The His_{10}-tagged protein was eluted at different concentrations of imidazole (25, 50, 300, and 500 mM) obtained by mixing buffer A with buffer B (buffer A with 500 mM imidazole). The enzymes were concentrated by ultrafiltration (Amicon YM 10 membrane; Millipore) and stored at −20°C with 50% (vol/vol) glycerol.

Molecular biological techniques. The *in silico* cloning steps were performed with the program Clone Manager 7.11 (Scientific & Educational Software). Standard protocols were used for purification, preparation, cloning, transformation, and amplification of DNA (19–21). Plasmid DNA isolation and purification of PCR products and plasmids were performed using Qiagen kits according to the manufacturer's specifications.

Calculation of *in vivo* specific carbon fixation rate of *B. xenovorans*. Specific growth rate (μ) was calculated from the generation time of the culture. The specific substrate (*S*) consumption (dS) per time unit (dt) was calculated as $dS/dt = (\mu/Y) \cdot X$, where *Y* represents the growth yield (1 g of dry cell mass formed per 0.5 g of carbon fixed) and *X* represents the cell dry mass in grams (1 g of cell dry mass, corresponding to approximately 0.5 g of protein).

Other methods. DNA sequence determination of purified plasmids was performed by GATC Biotech (Constance, Germany). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 12.5%) was performed using the Laemmli method (22). Proteins were visualized by Coomassie blue staining (23). Protein concentration was measured according to the Bradford method (24), using bovine serum albumin (BSA) as a standard. The purity of the enzymes was calculated by determining band intensities on an SDS gel using a ChemiDoc XRS+ imaging system and Image Lab software (version 3.0) (both from Bio-Rad).

RESULTS AND DISCUSSION

Growth of *B. xenovorans* on itaconate and mesaconate. The presence of the *P. aeruginosa*-like itaconate utilization gene cluster in the genome of *B. xenovorans* (Fig. 2) suggests that this bacterium may be able to utilize itaconate. Indeed, *B. xenovorans* grew

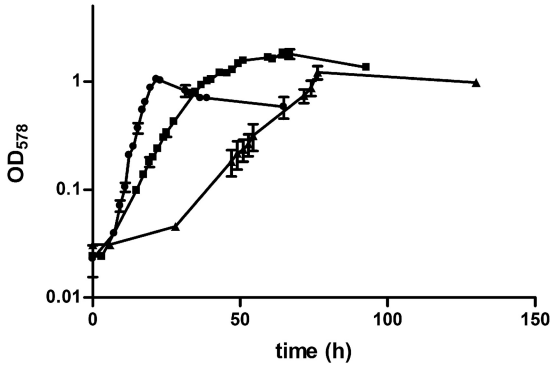

FIG 3 Growth of *B. xenovorans* on acetate (●), itaconate (■), and mesaconate (▲). The experiment was done in triplicate, with the error bars representing the standard deviations.

TABLE 2 Activities of enzymes involved in itaconate, (*S*)-citramalate, and mesaconate degradation in *B. xenovorans* cells grown on different substrates

Enzyme	Activity (nmol min^{-1} mg protein^{-1}) on growth substrate[a]:		
	Acetate	Itaconate	Mesaconate
Succinyl-CoA:itaconate-CoA transferase	26 ± 2	358 ± 68	168 ± 15
Itaconyl-CoA hydratase	2 ± 1	229 ± 42	13 ± 3
(*S*)-Citramalyl-CoA lyase	8 ± 2	680 ± 70	306 ± 52
Succinyl-CoA:(*S*)-citramalate-CoA transferase	<1	355 ± 98	181 ± 22
Fumarase	1,480 ± 140	290 ± 100	723 ± 83
Mesaconase	472 ± 46	113 ± 24	214 ± 27

[a] Values are means ± standard deviation of results from at least two independent measurements.

with itaconate as a sole carbon and energy source, although the growth was lower than with acetate (generation times of 6.4 and 2.8 h, respectively). Furthermore, *B. xenovorans* grew on mesaconate with a generation time of 9.7 h (Fig. 3).

Enzyme activities in *B. xenovorans*. Cell extracts of itaconate-grown *B. xenovorans* were capable to convert itaconate into acetyl-CoA and pyruvate (Tables 1 and 2; also see Fig. S1 in the supplemental material). The conversion was dependent either on succinyl-CoA or, 2.5 times less efficiently, on ATP and CoA (Table 1). Similar results were obtained for the conversions of mesaconate and (*S*)-citramalate into acetyl-CoA and pyruvate: they required succinyl-CoA as a CoA donor (Table 1; see also Fig. S1 in the supplemental material). Interestingly, no conversion was detected if citramalate and mesaconate were incubated with ATP and CoA (Table 1). This can likely be explained by the properties of succinyl-CoA synthetase, which is probably responsible for ATP-dependent activation of itaconate due to its promiscuity (13, 25) but is not active with (*S*)-citramalate and mesaconate (13). Therefore, the presence of succinyl-CoA as a CoA donor in a CoA transferase reaction is necessary for the utilization of these compounds (see below).

While itaconyl-CoA was detected as the major intermediate of itaconate conversion in the presence of succinyl-CoA (see Fig. S1 in the supplemental material), tiny amounts of citramalyl-CoA and mesaconyl-CoA were also detected in the reaction mixture (see Fig. S2 in the supplemental material). The conversion of (*S*)-citramalate into acetyl-CoA and pyruvate exhibited citramalyl-CoA as the main intermediate (see Fig. S1 and S2 in the supplemental material). Interestingly, no mesaconyl-CoA was detected when mesaconate was incubated with succinyl-CoA. The possible explanation of these data is that mesaconate utilization starts with its hydration to (*S*)-citramalate, which is then activated to (*S*)-citramalyl-CoA and cleaved into acetyl-CoA and pyruvate (Fig. 1; Table 2). This proposal is corroborated by the high similarity of *B. xenovorans* putative itaconate-CoA transferase Bxe_B2583 to the characterized itaconate-CoA transferase from *P. aeruginosa* (71% sequence similarity). Indeed, the *P. aeruginosa* enzyme is able to activate itaconate and (*S*)-citramalate but not mesaconate (11). Besides itaconate-CoA transferase, high activities of itaconyl-CoA hydratase and (*S*)-citramalyl-CoA lyase were detected in cell extracts of itaconate-grown *B. xenovorans* (Table 2). In contrast, the activities of all enzymes involved in itaconate utilization were substantially downregulated in acetate-grown cells, implying that the corresponding enzymes are substrate inducible.

The succinyl-CoA-dependent activity of itaconate conversion into acetyl-CoA and pyruvate in cell extracts of mesaconate-grown *B. xenovorans* was significantly lower than in itaconate-grown cells, whereas the activities of mesaconate and (*S*)-citramalate conversion were about the same in the two cases (Table 1; see also Fig. S3 in the supplemental material). Enzymatic assays revealed itaconyl-CoA hydratase reaction as a bottleneck in the conversion: its activity was an order of magnitude lower than the activities of itaconate-CoA transferase and (*S*)-citramalyl-CoA lyase (Table 2). Correspondingly, itaconyl-CoA accumulated in the reaction mixture (see Fig. S4 in the supplemental material). Although the differences in the activities of the itaconate degradation enzymes in the mesaconate-grown cells could be explained by the differences in the regulation of their genes, it cannot be excluded that a different set of genes encoding CoA transferase and (*S*)-citramalyl-CoA lyase is involved in the mesaconate catabolism.

The measured enzyme activities were sufficient to explain the observed growth rates. The *B. xenovorans* generation time during growth on mesaconate (9.7 h) corresponds to a specific carbon fixation rate *in vivo* of 93 nmol min^{-1} mg protein^{-1}. During aerobic growth on glucose, ~70% of the substrate is used as a carbon source (26). As the amount of ATP required for cell synthesis is

TABLE 1 Activities of itaconate, (*S*)-citramalate, mesaconate, and citraconate conversions into acetyl-CoA and pyruvate in extracts of *B. xenovorans* cells grown on different substrates

Reaction substrates	Activity (nmol min^{-1} mg protein^{-1}) on growth substrate[a]:		
	Acetate	Itaconate	Mesaconate
Itaconate + succinyl-CoA	1.0 ± 0.4	266 ± 1	11 ± 2
(*S*)-Citramalate + succinyl-CoA	1.3 ± 0.4	396 ± 18	224 ± 1
Mesaconate + succinyl-CoA	<1	35 ± 5	76 ± 5
Citraconate + succinyl-CoA	<1	<1	<1
Itaconate + ATP + CoA	2 ± 0.9	109 ± 22	17 ± 1
(*S*)-Citramalate + ATP + CoA	<1	<1	<1
Mesaconate + ATP + CoA	<1	<1	<1

[a] The activities were measured spectrophotometrically following pyruvate formation with phenylhydrazine. Values are means ± standard deviations of results from at least two independent measurements.

TABLE 3 Catalytic properties of recombinant fumarases from *B. xenovorans*[a]

Enzyme	Substrate	V_{max} (U mg protein^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} s^{-1})
Class I fumarase (Bxe_A3136)	Fumarate	296 ± 5	0.1 ± 0.01	2.8×10^6
	(*S*)-Malate	118 ± 3	0.28 ± 0.02	3.98×10^5
	Mesaconate	117 ± 6	0.03 ± 0.005	3.6×10^6
	(*S*)-Citramalate	74 ± 2	0.52 ± 0.05	1.31×10^5
Class II fumarase (Bxe_A1038)	Fumarate	376 ± 12	0.138 ± 0.013	2.2×10^6
	(*S*)-Malate	165 ± 6	0.42 ± 0.06	3.2×10^5
	Mesaconate	<0.1		
	(*S*)-Citramalate	<0.1		

[a] Values are means ± standard deviation of results from at least three measurements.

roughly inversely proportional to the number of carbon atoms of the carbon source with similar redox state (26), we propose that ~60% of mesaconate is probably used for anabolism and 40% for catabolism. Taking this assumption into account and because mesaconate is a C_5 compound, the minimal *in vivo* specific activity of enzymes involved in mesaconate conversion to acetyl-CoA and pyruvate can roughly be estimated around 30 nmol min^{-1} mg protein^{-1}. The activities of enzymes involved in mesaconate utilization in mesaconate-grown cells are considerably higher than this threshold.

Although mesaconase activity was required only in mesaconate-grown cells, it was regulated together with fumarase activity, constituting 30 to 40% of the latter (Table 2). This suggests that the corresponding enzyme is not encoded by the itaconate utilization gene cluster and that the mesaconase activity may be due to the promiscuity of one of the enzymes involved in central carbon metabolism. What kind of enzyme may catalyze the hydration of mesaconate into (*S*)-citramalate?

***B. xenovorans* class I fumarase is a promiscuous fumarase/mesaconase.** Fumarases catalyze the fumarate hydration in the citric acid cycle, which is very similar to the mesaconase (methylfumarase) reaction. Therefore, fumarase is an obvious candidate to catalyze the mesaconase reaction in *B. xenovorans*. *B. xenovorans* LB400 possesses two genes encoding fumarases, one belonging to the iron-dependent class I fumarases (Bxe_A3136) and the other belonging to the iron-independent class II fumarases (Bxe_A1038). The enzymes of these two classes are phylogenetically unrelated. Class I fumarases are thermolabile homodimeric proteins containing an FeS cluster, whereas class II fumarases are thermostable homotetramers with no requirements for metals or cofactors (27). To test the activity of *B. xenovorans* fumarases with mesaconate, we cloned the corresponding genes in pET16b vector and heterologously expressed them in *E. coli*. The resulting proteins were aerobically purified using a Ni-NTA column. SDS-PAGE analysis revealed the presence of a major band with an apparent molecular mass of 60 kDa (for class I enzyme) and 50 kDa (for class II fumarase) in the 300 mM imidazole fractions (see Fig. S5 in the supplemental material), which were further used to test the catalytic properties of the enzymes.

The class II fumarase Bxe_A1038 catalyzed the hydration of fumarate to (*S*)-malate as well as the reverse dehydration reaction, whereas no activity with either mesaconate or (*S*)-citramalate was detected (Table 3). The enzyme remained stable under aerobic conditions, and the incubation with Fe^{2+} and DTT did not result in the increase of its activity. Both K_m and V_{max} values of the enzyme were close to the published values for other class II fumarases (28, 29).

In contrast to the class II enzyme, the class I fumarase of *B. xenovorans*, Bxe_A3136, was oxygen sensitive, and the aerobically measured activity of the (aerobically) purified protein was relatively low. The incubation of the enzyme with Fe^{2+} and thiol and following measurement of the activity under strictly anaerobic conditions led to an ~4-fold increase in fumarate hydratase activity. Storage of the protein for 6 months led to almost complete loss of its activity, which, however, could be fully restored by the reactivation with Fe^{2+} and thiol. Therefore, the following enzyme characterization was performed with the reactivated enzyme.

The class I fumarase was highly active with fumarate and (*S*)-malate. The K_m and V_{max} values of this enzyme for fumarate and (*S*)-malate were similar to those of the class II enzyme (Table 3). However, the class I fumarase was also active with mesaconate and (*S*)-citramalate, whereas no activity was found with citraconate, (*R*)-citramalate, maleate, and (*R*)-malate. Moreover, the efficiency (k_{cat}/K_m) of the enzyme with mesaconate as the substrate was even slightly higher than with fumarate (Table 3), thus suggesting that *B. xenovorans* fumarase Bxe_A3136 is in fact a promiscuous fumarase/mesaconase. Interestingly, the K_m values for mesaconate hydration and (*S*)-citramalate dehydration in extracts of mesaconate-grown *B. xenovorans* cells (0.034 ± 0.006 and 0.47 ± 0.01 mM, respectively) were very similar to those of the heterologously produced class I fumarase (Table 3), further confirming the involvement of this enzyme in mesaconate utilization *in vivo*. These data disprove our previous suggestion (11) that the MmgE-PrpD family protein encoded in the itaconate utilization gene cluster of *P. aeruginosa* and *B. xenovorans* is responsible for mesaconate conversion into (*S*)-citramalate. Experimental identification of class I fumarase as mesaconase eliminates an orphan enzyme mentioned by The Orphan Enzyme Project (www.orphanenzymes.org) as (*S*)-2-methylmalate dehydratase (4.2.1.34) (30).

Mesaconase was first described in clostridia, where it takes part in the methylaspartate pathway of glutamate fermentation (7, 31). Furthermore, the participation of mesaconase in acetate assimilation in *Rhodospirillum rubrum* was proposed (32, 33). Mesaconase was also purified from different bacteria, and its presence was discussed in the context of the metabolism of various C_5-dicarboxylic acids (34–38). However, the gene encoding mesaconase has never been identified. Although the purified mesaconases from different sources had different subunit structures, all these enzymes were oxygen sensitive and could be reactivated with Fe^{2+} and a thiol. Furthermore, all characterized mesaconases accepted fumarate and (*S*)-malate as the substrates. Wang and Barker (39) already raised the question of whether fumarate and (*S*)-malate were substrates of mesaconase or whether this enzyme was contaminated with fumarase. In this study, we showed that mesaconate and (*S*)-citramalate are indeed substrates of a class I fumarase and that mesaconases may be fumarases or enzymes that are (phylogenetically) closely related to them. Class I fumarases occur in *Archaea* and *Bacteria*, including *E. coli* (fumarases A and B), as well as in many *Eukarya*, but not in mammals and *Saccharomyces cerevisiae* (27, 40–43). Whether the capability to hydrate mesaconate is an intrinsic property of all class I fumarases and whether other enzymes (apart from class I fumarases) are able to catalyze the mesaconase reaction are questions for further investigation.

C_5-dicarboxylic acids like mesaconic and methylsuccinic acids are regarded as important platform chemicals (44). Although they are commercially unavailable in bulk quantities to date (45, 46), their bioproduction is currently optimized (44, 46). The discovery of possible intrinsic mesaconase activity of class I fumarases should be taken into account in these studies, as the presence of class I fumarase may considerably influence mesaconate production. In addition, our findings open a way to synthesize (*S*)-citramalate from mesaconate using fumarate hydratase in a one-step process.

Conclusions. Here, we show that *B. xenovorans* class I fumarase is a promiscuous fumarase/mesaconase. This promiscuity is physiologically relevant, as it allows the growth of this bacterium on mesaconate as a sole carbon and energy source. The functioning of class I fumarase as mesaconase *in vivo* is supported by the coregulation of mesaconase and fumarase activities and by the similarities in K_m values for mesaconate and (*S*)-citramalate measured with cell extracts and with the purified class I fumarase as well as by the absence of other obvious candidate genes for mesaconase. The catalytic properties of the enzyme for fumarate and mesaconate are comparable, which may be a consequence of the similarity of these two compounds. Still, the findings document an adaptation of this enzyme (in contrast to the class II enzyme) for the utilization of mesaconate, which apparently occurs as a natural carbon source in the environment of *B. xenovorans*.

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