Characterization of the *mbd* cluster encoding the anaerobic 3-methylbenzoyl-CoA central pathway

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Running title: Anaerobic 3-methylbenzoate degradation

This article is dedicated to Kenneth N. Timmis for his seminal contributions and enthusiastic support to the field of environmental microbiology. E. Díaz wants to highlight the encouragement and advice of Ken along his scientific career.

Summary

The *mbd* cluster encoding genes of the 3-methylbenzoyl-CoA pathway involved in the anaerobic catabolism of 3-methylbenzoate and *m*-xylene was characterized for the first time in the denitrifying β-Proteobacterium *Azoarcus* sp. CIB. The *mbdA* gene product was identified as a 3-methylbenzoate-CoA ligase required for 3-methylbenzoate activation; its substrate spectrum was unique in activating all three methylbenzoate isomers. An inducible 3-methylbenzoyl-CoA reductase (*mbdONQP* gene products), displaying significant amino acid sequence similarities to known class I benzoyl-CoA reductases catalyzed the ATP-dependent reduction of 3-methylbenzoyl-CoA to a methyldienoyl-CoA. The *mbdW* gene encodes a methyldienoyl-CoA hydratase that hydrated the methyldienoyl-CoA to a methyl-6-hydroxymonoenoyl-CoA compound. The *mbd* cluster also contains the genes predicted to be involved in the subsequent steps of the 3-methylbenzoyl-CoA pathway as well as the electron donor system for the reductase activity. Whereas the catabolic *mbd* genes are organized in two divergent inducible operons, the putative *mbdR* regulatory gene was transcribed separately and showed constitutive expression. The efficient expression of the *mbd* genes required the oxygen-dependent AcpR activator, and it was subject of carbon catabolite repression by some organic acids and amino acids. Sequence analyses suggest that the *mbd* gene cluster was recruited by *Azoarcus* sp. CIB through horizontal gene transfer.

Introduction

Aromatic compounds constitute a structurally diverse and widespread class of organic compounds in nature. Moreover, a significant number of man-made environmental pollutants are also aromatic compounds (Rieger *et al.*, 2002; Boerjan *et al.*, 2003; Fuchs *et al.*, 2011). Microorganisms play an essential role in recycling carbon and maintaining the natural biogeochemical cycles in the biosphere. Bacteria have evolved to degrade most naturally occurring organic compounds, including the persistent aromatics (Lovley, 2003; Díaz, 2004; Fuchs, 2008; Fuchs *et al.*, 2011). There are two major strategies to degrade aromatic compounds depending on the presence or absence of oxygen (Fuchs, 2008; Carmona *et al.*, 2009; Fuchs *et al.*, 2011). Under anoxic conditions monoaromatic compounds are channeled via different peripheral pathways to a few central aromatic intermediates, e.g., resorcinol (1,3-dihydroxybenzene), phloroglucinol (1,3,5-trihydroxybenzene), hydroxyhydroquinone (1,2,4-trihydroxybenzene), benzoyl-CoA, and benzoyl-CoA analogues (Gibson and Harwood, 2002; Fuchs, 2008; Carmona *et al.*, 2009; Fuchs *et al.*, 2011). The degradation of many monoaromatic compounds leads to benzoyl-CoA as central intermediate and, therefore, the benzoyl-CoA pathway is the best characterized in facultative and strict anaerobes, usually grown in benzoate as carbon source (Harwood *et al.*, 1999; Boll, 2005; Fuchs, 2008; Carmona *et al.*, 2009; Fuchs *et al.*, 2011). This catabolic pathway starts with the activation of benzoate to benzoyl-CoA by a benzoate-CoA ligase (Fuchs, 2008; Carmona *et al.*, 2009; Fuchs *et al.*, 2011). Due to the full aromatic character of benzoyl-CoA, its reduction is mechanistically difficult to achieve and requires a low-potential electron donor ferredoxin, and the strictly anaerobic benzoyl-CoA reductase (BCR) reaction has to be coupled to an exergonic one (Boll, 2005; Fuchs *et al.*, 2011). In facultative anaerobes and some strict anaerobes, as in the archaeon *Ferroglobus*

placidus, there is a class I BCR that requires ATP for the irreversible transfer of electrons to the aromatic ring. Class I BCR complexes are composed of four subunits and accept electrons from a reduced ferredoxin protein (Boll, 2005; Fuchs *et al.*, 2011; Holmes *et al.*, 2012). However, in most strict anaerobes, a class II BCR catalyzes the ATP-independent and reversible reduction of the aromatic ring that may be driven by a membrane potential (Kung *et al.*, 2009; Löffler *et al.*, 2010). Both classes of BCRs produce an alicyclic compound, usually cyclohex-1,5-diene-1-carbonyl-CoA (dienoyl-CoA), but in *Rhodopseudomonas palustris* (and probably *F. placidus*) strains the reduction product is cyclohex-1-ene-1-carbonyl-CoA. The latter is then metabolized by a modified β-oxidation reaction sequence, i.e., hydration of a double bond, dehydrogenation and hydrolytic ring cleavage, finally generating an aliphatic C_7-dicarboxyl-CoA derivative (Laempe *et al.*, 1998; Laempe *et al.*, 1999; Gibson and Harwood, 2002; Fuchs, 2008; Carmona *et al.*, 2009; Fuchs *et al.*, 2011). Further degradation of the C_7-dicarboxyl-CoA derivative through conventional β-oxidation-like reactions via glutaryl-CoA and crotonyl-CoA yields three acetyl-CoAs and one CO_2 (lower benzoyl-CoA pathway) (Harwood, *et al.*, 1999; Harrison and Harwood, 2005; Carmona *et al.*, 2009; Fuchs *et al.*, 2011). The gene clusters responsible for the upper benzoyl-CoA pathway have been described in different facultative and obligate anaerobes that belong to different subgroups of Proteobacteria, e.g. *Rhodopseudomonas* and *Magnetospirillum* strains (α-Proteobacteria), *Thauera* and *Azoarcus/"Aromatoleum"* strains (β-Proteobacteria), *Geobacter, Syntrophus* and the NaphS2 sulfate reducer strains (δ-Proteobacteria) (Carmona *et al.*, 2009; DiDonato *et al.*, 2010), as well as in the hyperthermophilic archaeon *F. placidus* (Holmes *et al.*, 2012). The expression of the catabolic genes is transcriptionally controlled by specific regulators that are encoded in the cognate benzoyl-CoA catabolic clusters, e.g., the

BzdR regulator in *Azoarcus* sp. CIB (Durante-Rodríguez *et al*., 2010), the BadR and BadM regulators in *Rhodopseudomonas palustris* (Egland and Harwood, 1999; Peres and Harwood, 2006), and the BgeR and BamVW two-component regulatory system in *Geobacter* strains (Juárez *et al*., 2010; Ueki, 2011). Moreover, there is an additional level or regulation mediated by global regulators, e.g., the AadR and AcpR oxygen-responding Fnr-like regulatory proteins, that adjust the specific transcriptional regulation to the physiological and metabolic state of the cells (Dispensa *et al.*, 1992; Durante-Rodríguez *et al.*, 2006; Carmona *et al.*, 2009).

Several benzoyl-CoA analogues have been reported as intermediates in the anaerobic degradation of substituted benzoates such as 3-hydroxybenzoate, 2-aminobenzoate and 3-chlorobenzoate. Whereas a modified benzoyl-CoA central pathway has been described for the anaerobic mineralization of 3-hydroxybenzoyl-CoA (Laempe *et al.*, 2001; Wöhlbrand *et al*., 2007), 3-chlorobenzoyl-CoA is directly converted to benzoyl-CoA by a class I BCR and degraded by the classical benzoyl-CoA pathway (Kuntze *et al*., 2012). Alkylbenzenes, such as toluene and *p*-xylene, are channeled through a common peripheral pathway to the benzoyl-CoA and 4-methylbenzoyl-CoA central pathways, respectively (Heider, 2007; Lahme *et al*., 2012). The initial anaerobic degradation of *m*-xylene resembles that of toluene since it seems to be catalyzed by the same enzymes through the addition of the methyl moiety to fumarate (Heider, 2007; Boll and Heider, 2010). However, whereas toluene generates benzoyl-CoA, *m*-xylene generates 3-methylbenzoyl-CoA as final product of the anaerobic peripheral pathway (Krieger *et al*., 1999; Achong *et al*., 2001; Morasch *et al.*, 2004; Hermann *et al*. 2009; Rabus *et al.* 2011). *o*-Cresol is also metabolized in some anaerobes by carboxylation, CoA activation and reductive dehydroxylation to 3-methylbenzoyl-CoA (Rudolphi *et al.*, 1991). Moreover, the anaerobic catabolism of 3-

methylbenzoate also involves its activation to 3-methylbenzoyl-CoA. Thus, 3-methylbenzoyl-CoA behaves as a central intermediate formed during the anaerobic catabolism of different aromatic compounds. Despite several bacteria are able to degrade benzoate anaerobically, only a few of them can use 3-methylbenzoate as sole carbon source, suggesting that the latter is not degraded via the classical benzoyl-CoA pathway (Song *et al*., 2001; Carmona *et al*., 2009), but rather through a new central pathway that has not been studied so far.

Azoarcus sp. CIB is a denitrifying β-Proteobacterium able to anaerobically degrade different aromatic compounds, including some hydrocarbons such as toluene, via benzoyl-CoA (López-Barragán *et al*., 2004a; Blázquez *et al*., 2008). The *bzd* gene cluster responsible of the benzoyl-CoA central pathway is organized as a single catabolic operon and a *bzdR* regulatory gene. The expression of the *bzd* genes is controlled by the BzdR transcriptional repressor and benzoyl-CoA is the inducer molecule (Barragán *et al*., 2005; Durante-Rodríguez *et al*., 2010). A benzoate-CoA ligase activity, encoded by the *bzdA* gene, and a BCR activity, likely encoded by the *bzdNOPQ* genes, have been described in strain CIB (López-Barragán *et al*., 2004a). The *Azoarcus* sp. CIB strain is also able to grow anaerobically using *m*-xylene as sole carbon source, and we have reported some mutant strains that lost the ability to use toluene/benzoate but retained the ability to use *m*-xylene, suggesting the existence of a catabolic pathway, other than the bzd pathway, for the anaerobic catabolism of *m*-xylene (Blázquez *et al*., 2008). In this work, we have identified and functionally characterized the gene cluster responsible for the 3-methylbenzoyl-CoA central pathway in *Azoarcus* sp. CIB.

Results and discussion

Identification of the mbd gene cluster responsible for the anaerobic catabolism of 3-methylbenzoate in Azoarcus sp. CIB

As indicated in the Introduction, 3-methylbenzoyl-CoA is a common intermediate formed during the anaerobic degradation of some aromatic compounds such as *m*-xylene and 3-methylbenzoate. Since *Azoarcus* sp. CIB is able to use *m*-xylene under anaerobic conditions, we firstly checked whether the strain was also able to degrade 3-methylbenzoate anaerobically. As expected, *Azoarcus* sp. CIB was able to grow anaerobically in 3-methylbenzoate (3 mM) under batch conditions, although with a doubling time (16-24 h) higher than that shown in benzoate (6-10 h). On the other hand, the *Azoarcus* sp. CIBd*bzdN* strain, which contains an inactive *bzd* gene cluster and therefore is unable to use benzoate anaerobically (López-Barragán *et al*., 2004a), retained the ability to use 3-methylbenzoate as sole carbon and energy source (data not shown), indicating that the anaerobic degradation of benzoate and 3-methylbenzoate follow different central pathways. Analysis of the *Azoarcus* sp. CIB genome revealed the presence of a 29.7-kb chromosomal region, hereafter referred to as *mbd* (3-methylbenzoate degradation) gene cluster, that is located adjacent to the cluster encoding the peripheral pathway for anaerobic toluene/*m*-xylene degradation (Fig. 1). An overall analysis of the *mbd* cluster revealed 26 open reading frames, most of which encoded proteins that showed a significant identity with enzymes and transporters involved in the central pathways for the anaerobic degradation of aromatic acids such as benzoate (Egland *et al*., 1997; Breese *et al*., 1998; López-Barragán *et al*., 2004a; López-Barragán *et al.*, 2004b; Shinoda *et al*., 2005; Rabus *et al*., 2005; Wischgoll *et al*., 2005; McInerney *et al.*, 2007; DiDonato *et al.*, 2010; Holmes *et al*., 2012; Lahme *et al*., 2012), 3-hydroxybenzoate (Laempe *et al.*, 2001; Wöhlbrand *et al*., 2007), and 4-

methylbenzoate (Lahme *et al*., 2012) (Table 1). Amino acid sequence comparison analyses between the Mbd proteins and their Bzd orthologs in *Azoarcus* sp. CIB revealed levels of identity ranging from 24% (MbdO/BzdO) to 47% (MbdA/BzdA). The chromosomal location and the sequence comparison analyses suggested, therefore, that the *mbd* cluster could encode a new central pathway for the anaerobic degradation of benzoate derivatives, most likely 3-methylbenzoate via 3-methylbenzoyl-CoA, the product of the *m*-xylene peripheral pathway (Krieger *et al*., 1999; Achong *et al*., 2001; Morasch *et al.*, 2004; Hermann *et al*. 2009; Rabus *et al.* 2011). To confirm this assumption, we constructed *Azoarcus* sp. CIB mutant strains with insertional disruptions within some of the genes of the *mbd* cluster. The strains *Azoarcus* sp. CIBd*orf6*, CIBd*orf5*, CIBd*mbdY*, CIBd*mbdO*, and CIBd*mbdA*, with disruptional insertions within the *orf6, orf5, mbdY, mbdO* and *mbdA* genes, respectively, were unable to grow anaerobically in 3-methylbenzoate but they retained the ability to use benzoate as sole carbon source (Table 2). These results demonstrate that the anaerobic degradation of benzoate and 3-methylbenzoate follow different central pathways, and that the *mbd* cluster is involved in the anaerobic degradation of the latter compound in *Azoarcus* sp. CIB. Moreover, the *Azoarcus* sp. CIBd*orf6*, CIBd*orf5*, CIBd*mbdY*, and CIBd*mbdO* strains were unable to use *m*-xylene but they used toluene as sole carbon source (Table 2), which is in agreement with the fact that the anaerobic catabolism of *m*-xylene and toluene generates 3-methylbenzoyl-CoA and benzoyl-CoA, respectively, as central intermediates (Krieger *et al*., 1999; Achong *et al*., 2001; Morasch *et al.*, 2004; Hermann *et al*. 2009; Rabus *et al.* 2011).

The mbdA gene encodes the 3-methylbenzoate-CoA ligase

Azoarcus sp. CIBd*mbdA*, a strain that has a disrupted *mbdA* gene, was unable to degrade 3-methylbenzoate but it used *m*-xylene as sole carbon source (Table 2),

indicating that the *mbdA* gene is not essential for the anaerobic catabolism of *m*-xylene. On the other hand, the *mbdA* gene product shows a significant amino acid sequence identity with aromatic acid-CoA ligases (Table 1), suggesting that it corresponds to the 3-methylbenzoate-CoA ligase required for the initial activation of 3-methylbenzoate to 3-methylbenzoyl-CoA, a metabolic step that is dispensable to degrade the 3-methylbenzoyl-CoA generated directly from the *m*-xylene peripheral pathway (Krieger *et al.*, 1999).

The *mbdA* gene from *Azoarcus* sp. CIB was PCR amplified and cloned under control of the *Plac* promoter into a pUC19 vector, producing plasmid pUCmbdA (Table 2). Cell extracts from an *Escherichia coli* DH10B strain harboring plasmid pUCmbdA that expressed the MbdA protein showed 3-methylbenzoate-CoA ligase activity (0.31 μmol min^{-1} mg of $protein^{-1}$), and this activity could not be detected with the *E. coli* DH10B strain harboring the control plasmid pUC19. This result indicates that the *mbdA* gene indeed encodes a 3-methylbenzoate-CoA ligase. The substrate specificity of MbdA revealed that this enzyme was also able to activate benzoate with an activity similar to that shown with 3-methylbenzoate (Fig. 2). Aromatic compounds with a longer carboxylic chain, e.g., phenylacetate or phenylpropionate, were not substrates of the MbdA enzyme. The change of the methyl group from the *meta* position to the *ortho* (2-methylbenzoate) or *para* (4-methylbenzoate) position reduced the MbdA activity to a 50% and 30% of that shown with 3-methylbenzoate (Fig. 2). Some halogenated derivatives of benzoate were suitable substrates for MbdA, specially when the halogen group was located in *meta* position, e.g., 3-chlorobenzoate and 3-fluorobenzoate, giving rise to 77% and 40% of the activity observed with 3-methylbenzoate, respectively. However, replacement of the methyl group by a polar one, such as in 3-hydroxybenzoate or 3-hydroxyphenylpropionate, did not allow CoA ligase activity by

the MbdA enzyme (Fig. 2). In summary, the MbdA ligase represents the first aromatic acid CoA ligase described so far that is shown to use the three methylbenzoate isomers as substrates. MbdA becomes an enzyme of potential biotechnological interest for the enzymatic synthesis of methylbenzoyl-CoA compounds that could be used as substrates in enzymatic benzoylations for the synthesis of different compounds some of which, e.g., Taxol variants, are of pharmaceutical interest (Beuerle and Pichersky, 2002; Nawarathne and Walker, 2010).

Phylogenetic analysis of the reported benzoate-CoA ligases and CoA ligases that act on some aromatic-ring substituted benzoate analogues reveals that they cluster into several main groups, e.g., benzoate-, hydroxybenzoate-, aminobenzoate-, and 4-methylbenzoate-CoA ligases, being MbdA the first member of a new evolutionary branch within the benzoate-CoA ligase group (Fig. S1).

In some denitrifying bacteria, such as *Thauera* and *Magnetospirillum* strains (Schüle *et al*., 2003; Kawaguchi *et al*., 2006), the same benzoate-CoA ligase may be shared by different degradation pathways, e.g., BclA activates benzoate aerobically and anaerobically but also catalyses 2-aminobenzoyl-CoA formation in *T. aromatica* (Schüle *et al*., 2003). In contrast, in other denitrifying microorganisms, such as *Azoarcus/"Aromatoleum"* strains (Rabus *et al*., 2005; Carmona *et al*., 2009), as well as in the phototroph *R. palustris* (Egland *et al*., 1995), every catabolic cluster appears to have its own specific CoA ligase gene, e.g., the *bzdA* and *bclA* genes from the anaerobic and aerobic benzoate degradation clusters, respectively (López-Barragán *et al.*, 2004a; Valderrama *et al*., 2012), and the *mbdA* gene from the anaerobic 3-methylbenzoate degradation cluster in *Azoarcus* sp. CIB. Thus, different genetic strategies are used for the initial activation of the aromatic compounds during their anaerobic degradation by closely related microorganisms.

Identification of the 3-methylbenzoyl-CoA reductase activity

The amino acid sequence analysis of the *mbdONQP* gene products shows that they have significant similarities with the β, γ, α and δ subunits, respectively, of the class I BCRs from *T. aromatica* and *R. palustris* strains (Table 1) (Boll, 2005), suggesting that they constitute the four subunits of a 3-methylbenzoyl-CoA reductase that catalyzes the second step of the 3-methylbenzoate degradation pathway. To explore further this assumption, *Azoarcus* sp. CIB and *Azoarcus* sp.CIBd*mbdO* cells were grown anaerobically in glutarate plus 3-methylbenzoate, and the 3-methylbenzoyl-CoA reductase activity was assayed in cell extracts by HPLC analysis following the conversion of synthesized 3-methylbenzoyl-CoA (Fig. 3A). Whereas *Azoarcus* sp. CIB cell extracts showed a specific activity of 3-methylbenzoyl-CoA conversion of 7.5 nmol min^{-1} mg $protein^{-1}$, *Azoarcus* sp. CIBd*mbdO* cell extracts showed no detectable reductase activity (< 0.1 nmol min^{-1} mg $protein^{-1}$) (Table 3). Moreover, the 3-methylbenzoyl-CoA reductase activity appeared to be inducible, as *Azoarcus* sp. CIB cells grown anaerobically on glutarate in the absence of 3-methylbenzoate showed less than 1% of the activity calculated for cells grown in the presence of 3-methylbenzoate (Table 3). These results confirm the existence of an inducible 3-methylbenzoyl-CoA reductase activity in *Azoarcus* sp. CIB, and strongly suggest that the *mbdONQP* genes are responsible for this activity. Based on the typical retention times of the dienoyl-CoA/benzoyl-CoA compounds (Fig. 3), and their characteristic UV/VIS spectra (Fig. S2), the product from 3-methylbenzoyl-CoA most probably represents a methylated cyclohex-1,5-diene-1-carbonyl-CoA analogue (methyldienoyl-CoA) with a typical absorption shoulder at 310 nm. This finding indicates a two electron reduction of 3-methylbenzoyl-CoA similar to that reported for benzoyl-CoA by most BCRs (Boll, 2005), yielding either 3-methyldienoyl-CoA or 5-methyldienoyl-CoA (Fig. 1).

The previously reported BCR activity induced in *Azoarcus* sp. CIB cells grown anaerobically in benzoate (López-Barragán *et al.*, 2004a), was also observed by HPLC analysis in this work (Table 3). Interestingly, *Azoarcus* sp. CIB cells grown anaerobically in 3-methylbenzoate were also able to reduce benzoyl-CoA (Fig. 3B and Table 3), and cells grown on benzoate were able to reduce 3-methylbenzoyl-CoA (Table 3), suggesting that both reductases were able to catalyze both aryl-CoA conversions. To confirm this hypothesis, we checked the reductase activity present in cells that contain only one of the two reductases, i.e., *Azoarcus* sp. CIBd*mbdO* cells, that contain an inactive 3-methylbenzoyl-CoA reductase, and *Azoarcus* sp. CIBd*bzdN* cells, that contain an inactive BCR (López-Barragán *et al*., 2004a). Since *Azoarcus* sp. CIBd*mbdO* cells grown in benzoate and *Azoarcus* sp. CIBd*bzdN* cells grown in 3-methylbenzoate were capable to reduce both 3-methylbenzoyl-CoA and benzoyl-CoA (Table 3), these results strongly suggest that BCR and the 3-methylbenzoyl-CoA reductase from *Azoarcus* sp. CIB are isoenzymes that reduce both benzoyl-CoA and 3-methylbenzoyl-CoA. This finding agrees with earlier observations that purified BCR from *T. aromatica* was able to reduce 3-methylbenzoyl-CoA (Möbitz and Boll, 2002). The BCRs from *T. aromatica* and *Magnetospirillum* sp. strain pMbN1 do not use 4-methylbenzoyl-CoA as substrate, and a specific 4-methylbenzoyl-CoA reductase has been recently described in the latter strain (Lahme *et al*., 2012). Therefore, it appears that the existence of several gene clusters encoding different aryl-CoA reductases in a bacterial genome may respond to the different substrate specificity of the corresponding enzymes, e.g., benzoyl-CoA and 4-methylbenzoyl-CoA reductases, and/or to a differential induction of the genes encoding such enzymes when the cells grow in different substituted benzoates, e.g., benzoyl-CoA and 3-methylbenzoyl-CoA reductase isoenzymes.

Identification of the methyl-cyclohex-1,5-diene-1-carbonyl-CoA hydratase

The product of the BCR in denitrifying bacteria, i.e., the cyclohex-1,5-diene-1-carbonyl-CoA (dienoyl-CoA), is the substrate of a hydratase activity in the modified β-oxidation reaction scheme of the benzoyl-CoA upper pathway (Fuchs, 2008; Carmona *et al.*, 2009; Fuchs *et al.*, 2011). Since the product of the *mbdW* gene shows similarities with dienoyl-CoA hydratases (Table 1), MbdW appeared to be a suitable candidate to perform the predicted methyldienoyl-CoA hydratase activity in the mbd pathway (Fig. 1). To confirm this, the *mbdW* gene was PCR-amplified and cloned generating plasmid pETmbdW that expressed the MbdW protein with a His_6 tag at its C-terminus (Table 2). The MbdW protein was purified from the soluble protein fraction of *E. coli* BL21 (DE3) cells containing plasmid pETmbdW by a single-step Ni-chelating chromatography (Fig. S3). The oligomeric state of MbdW was analyzed by gel filtration chromatography and the calculated apparent molecular mass was 86 ± 9 kDa. As the molecular mass of the MbdW monomer predicted from the gene sequence is 29 kDa, the native conformation of the MbdW protein in solution could be a trimer, although a dimer cannot be discarded. An oligomeric conformation of either a dimer or trimer has been also reported for BamR, the cyclohex-1,5-diene-1-carbonyl-CoA hydratase implicated in the anaerobic degradation of benzoate in *G. metallireducens* (Peters *et al.*, 2007), being the dienoyl-CoA hydratase (Dch) from *T. aromatica* a dimer in solution (Laempe *et al.*, 1998).

The predicted hydratase activity of the MbdW protein was monitored in a discontinuous assay following substrate consumption and product formation by HPLC analysis. MbdW converted methyldienoyl-CoA to a more polar product whose characteristic retention time and UV/VIS spectrum corresponded to that of methylated 6-hydroxycyclohex-1-ene-1-carbonyl-CoA (methyl-6-hydroxymonoenoyl-CoA) (Fig. S4A). As already shown with other dienoyl-CoA hydratases (Laempe *et al.*, 1998;

Peters *et al.*, 2007), MbdW was able to catalyze the reverse reaction, i.e., the dehydration of methyl-6-hydroxymonoenoyl-CoA to methyldienoyl-CoA (Fig. 4A). Interestingly, MbdW was also able to efficiently hydrate/dehydrate the non-methylated derivatives formed in the classical benzoyl-CoA pathway, i.e., dienoyl-CoA (Fig. S4B) and 6-hydroxymonoenoyl-CoA (Fig. 4B). Using a spectrophotometric assay that monitors the dehydration of 6-hdyroxymonoenoyl-CoA to dienoyl-CoA, a rate of 478 μmol min^{-1} mg $protein^{-1}$ was determined for MbdW. A similar rate (463 μmol min^{-1} mg $protein^{-1}$) was obtained for the BamR hydratase from *Syntrophus aciditrophicus* (Peters *et al.*, 2007).

All these results indicate that MbdW is a hydratase that catalyzes the third step in the anaerobic 3-methylbenzoate degradation pathway, initiating the modified β-oxidation of the alicyclic methyldienoyl-CoA (Fig. 1).

Further degradation of methyl-6-hydroxymonoenoyl-CoA

The next steps for the conversion of methyl-6-hydroxymonoenoyl-CoA would consist in a dehydrogenation and hydrolysis reaction that would originate methyl-6-ketocyclohex-1-ene-1-carbonyl-CoA and finally 3-hydroxy-methyl-pimelyl-CoA, respectively (Fig. 1). The predicted proteins responsible for the dehydrogenation and hydrolysis activities of the 3-methylbenzoyl-CoA upper pathway are MbdX and MbdY, respectively, since they show a high similarity to the dehydrogenase (Had) and hydrolase (Oah) of the benzoyl-CoA central pathway in *T. aromatica* (Table 1) (Laempe *et al.*, 1999).

Degradation of the aliphatic product of the mbd upper pathway, i.e., 3-hydroxy-6-methyl-pimelyl-CoA or 3-hydroxy-4-methyl-pimelyl-CoA, could be accomplished via conventional β-oxidation of dicarboxylic acids (*pim* genes) (Harrison and Harwood,

2005; Carmona *et al*., 2009) with the formation of 4-methylglutaryl-CoA or 2-methylglutaryl-CoA, respectively (Fig. S5). The methylglutaryl-CoA appears to be degraded by a specific lower pathway since *Azoarcus* sp. CIBd*gcdH*, a strain that lacks the glutaryl-CoA dehydrogenase involved in the benzoyl-CoA lower pathway and, therefore, is unable to grow anaerobically in benzoate (Blázquez *et al*., 2008), grows in 3-methylbenzoate as sole carbon source (data not shown). Amino acid sequence comparison analyses (Table 1), and the observation that *Azoarcus* sp. CIB mutant strains harbouring disrupted *orf5* and *orf6* genes are unable to use 3-methylbenzoate anaerobically (Table 2), suggest that some of the *orf1-orf9* genes located within the *mbd* gene cluster (Fig. 1) could be involved in the specific 3-methylbenzoyl-CoA lower pathway capable of dealing with the *meta*-methyl group. Thus, Orf5 is predicted to be the methylglutaryl-CoA dehydrogenase that forms either 2-methylcrotonyl-CoA or 2-pentenoyl-CoA (Fig. S5). Finally, 2-methylcrotonyl-CoA or 2-pentenoyl-CoA could be further degraded to acetyl-CoA and propionyl-CoA by a β-oxidation reaction sequence analogous to that of the widespread isoleucine pathway (Conrad *et al*., 1974) or to the 5-aminovalerate pathway in *Clostridium aminovalericum* (Barker *et al*., 1987), respectively. Enzyme candidates for this reaction sequence could be Orf3/Orf9 (both predicted enoyl-CoA hydratases), Orf4 (predicted hydroxyacyl-CoA dehydrogenase), and Orf2 (predicted β-ketothiolase) (Table 1) (Fig. S5).

Transcriptional organization and induction of the mbd cluster

The gene organization within the *mbd* cluster in *Azoarcus* sp. CIB reveals that catabolic, transport and regulatory genes are arranged in at least three operons controlled by promoters located upstream of *mbdO* (P_O), *mbdB1* (P_{B1}) and *mbdR* (P_{3R}) genes (Fig. 5). To confirm the existence of the proposed operons, the co-transcription of the longer intergenic regions (*mbdW-mbdM*, 636 bp; *mbdP-orf1*, 621 bp; *orf5-orf6*, 481 bp;

mbdB5-mbdA, 231 bp; *mbdM-korA2*, 202 bp; *korB2-orf5*, 158 bp) was analyzed by RT-PCR experiments using total RNAs harvested from *Azoarcus* sp. CIB cells grown anaerobically in 3-methylbenzoate, succinate or benzoate as sole carbon sources. The analysis of the RT-PCR amplification products strongly suggests that the *mbdO-orf9* genes, and the *mbdB1-mbdA* genes are co-transcribed when *Azoarcus* sp. CIB was grown in 3-methylbenzoate and, therefore, they constitute a couple of divergent operons driven by the P_O and P_{B1} promoters, respectively (Fig. 5). Interestingly, the expression of the two divergent *mbd* operons was only observed when *Azoarcus* sp. CIB cells were grown in 3-methylbenzoate, but not when the cells were cultivated in succinate or benzoate (Fig. 5), indicating that the P_O and P_{B1} promoters of the *mbd* cluster are inducible and their activity depends on the 3-methylbenzoate metabolism. On the contrary, the putative *mbdR* regulatory gene is transcribed separately and the P_{3R} promoter was constitutively active in all the assayed conditions (Fig. 5). The differential expression of catabolic and regulatory genes is a common feature found in other aromatic degradation clusters (Carmona *et al*., 2009). The 3-methylbenzoate specific induction of the catabolic *mbd* genes is a key element in determining the growth capacity of *Azoarcus* sp. CIB, and explains why an *Azoarcus* sp. CIBd*bzdN* strain lacking a functional bzd pathway cannot use benzoate anaerobically despite the three first enzymes in the 3-methylbenzoate degradation pathway can also use benzoate and the corresponding benzoate products as substrates (see above).

Overimposed regulation of the mbd genes

The presence of oxygen and additional carbon sources in the media are two major environmental signals that have been shown to influence the expression of catabolic clusters for the anaerobic degradation of aromatic compounds in bacteria (Carmona *et al.*, 2009; Trautwein *et al.*, 2012). Regarding the oxygen-dependent expression of some

catabolic pathways, *Azoarcus* sp. CIB was shown to posses an Fnr-like transcriptional regulator encoded by the *acpR* gene that plays an essential role as a transcriptional activator of the *bzd* cluster responsible for the anaerobic degradation of benzoate (Durante-Rodríguez *et al.*, 2006) To check if AcpR could play a similar role in the expression of the *mbd* cluster, the ability of *Azoarcus* sp. CIBd*acpR*, a mutant strain that lacks a functional *acpR* gene (Durante-Rodríguez *et al.*, 2006), to grow in 3-methylbenzoate was checked. Whereas *Azoarcus* sp. CIBd*acpR* was unable to grow anaerobically in 3-methylbenzoate, the same strain harbouring the pIZ-FNR* plasmid, that expresses the Fnr* protein from *E. coli* (Durante-Rodríguez *et al.*, 2006) (Table 2), acquired the ability to use 3-methylbenzoate as sole carbon source (data not shown), suggesting the implication of the AcpR protein in the regulation of the *mbd* cluster. RT-PCR experiments confirmed that the expression of the *mbd* genes from the P_O, P_{B1} and P_{3R} promoters decreased in an *Azoarcus* sp. CIBd*acpR* strain with respect to their expression levels in the parental strain (Fig. 6). These results indicate that AcpR is not only an essential regulator that mediates the oxygen-dependent expression of the benzoyl-CoA central pathway (Durante-Rodríguez *et al.*, 2006; Carmona *et al.*, 2009), but also controls the anaerobic expression of other aromatic central pathways, e.g, the *mbd* genes, in *Azoarcus* sp. CIB.

It has been previously shown that the benzoyl-CoA pathway is subject of catabolite repression when *Azoarcus* sp. CIB cells grow anaerobically in the presence of benzoate plus an additional carbon source such as succinate, acetate or malate (López-Barragán *et al.*, 2004a). To check whether the 3-methylbenzoyl-CoA pathway is also under carbon catabolite control, we monitored the activity of the P_{B1} promoter by constructing plasmid pIZP$_{B1}$ that expresses the P_{B1}*::lacZ* reporter fusion (Table 2). The *Azoarcus* sp. CIB strain containing plasmid pIZP$_{B1}$ was grown anaerobically on

different carbon sources with or without the addition of 3-methylbenzoate, and β-galactosidase assays were performed when the cultures reached mid-exponential phase. Whereas pyruvate, cyclohexanecarboxylate and a mixture of all amino acids (casamino acids) caused a clear repressive effect on the activity of P_{B1}, benzoate, alanine and glutarate did not significantly affect the expression of the P_{B1}*::lacZ* fusion when compared with the expression observed using 3-methylbenzoate as sole carbon source (Fig. 7). These results strongly suggest that there is a repressive effect of some aliphatic/alicyclic organic acids and amino acids on the 3-methylbenzoyl-CoA pathway, and this effect is carried out at the level of transcription of the *mbd* genes. Further work is needed to determine whether the catabolite repression effect on the benzoyl-CoA and 3-methylbenzoyl-CoA pathways in *Azoarcus* sp. CIB follows a common regulatory mechanism, and which is the molecular basis underlying such catabolite repression control. In this sense, it is worth to mention that different regulatory strategies controlling the substrate utilization preferences may exist even among closely related strains since "*A. aromaticum*" EbN1 shows a preferred benzoate utilization from a mixture of C_4-dicarboxylates (succinate, fumarate, malate) and benzoate (Trautwein *et al.*, 2012).

Evolutionary considerations

Two subclasses of conventional class I BCRs have been described based on amino acid sequence comparison analyses, i.e., i) *Thauera*-subclass BCRs, that include the BCRs from *Thauera, Magnetospirillum* and *Rhodopseudomonas* strains, and ii) *Azoarcus*-subclass BCRs, that include the BCRs from *Azoarcus*/"*Aromatoleum*" strains and that predicted in *F. placidus*, as well as the putative 3-hydroxybenzoyl-CoA reductase from "*A. aromaticum*" EbN1 (Boll, 2005; Song and Ward, 2005; Rabus *et al.*, 2005; Carmona *et al.*, 2009; Holmes *et al.*, 2012). In this work, we show that the four subunits

of the 3-methylbenzoyl-CoA reductase from *Azoarcus* sp. CIB display varying degrees of relatedness with the subunits of conventional class I BCRs, i.e., whereas the MbdO (β subunit), MbdN (γ subunit) and MbdP (δ subunit) branch as a new subgroup within the *Thauera*-subclass of BCRs, the MbdQ (α subunit) branches as a new subgroup of the *Azoarcus*-subclass of BCRs (Fig. S6). Interestingly, the four subunits of the Mbd reductase cluster with the equivalent subunits of the recently described 4-methylbenzoyl-CoA reductase from *Magnetospirillum* sp. pMbN1 (Lahme *et al*., 2012), suggesting that methylbenzoyl-CoA reductases may have a common evolutionary origin (Fig. S6).

The *mbd* cluster also contains the *mbdM* and the *korA2B2* genes that encode proteins with significant similarity to the BCR-associated ferredoxin and the two-subunit 2-oxoglutarate:ferredoxin oxidoreductase (KGOR) that regenerates the reduced ferredoxin in *T. aromatica* (Dörner and Boll, 2002), respectively (Table 1 and Fig. 1). Similar genes involved in the regeneration of the reducing power needed for the BCR enzyme have been identified in *Magnetospirillum* strains (López-Barragán *et al*., 2004b; Shinoda *et al*., 2005; Lahme *et al*., 2012) (Fig. S7). In contrast, the primary electron donor of BCR in *Azoarcus/"Aromatoleum"* strains, the BzdM ferredoxin, clusters in a different phylogenetic branch than that of the *Thauera*-type ferredoxins (Fig. S7), and it is regenerated by the combined action of a three-subunit NADP-dependent KGOR enzyme that differs from the two-subunit *Thauera*-type KGOR enzyme (Ebenau-Jehle *et al*., 2003). These data strongly suggest that the electron donor system for the 3-methylbenzoyl-CoA reductase in *Azoarcus* sp. CIB belongs to the *Thauera*-type rather than to the *Azoarcus*-type, which is also in agreement with the fact reported above that the reductase enzyme does not belong to the *Azoarcus*-subclass of BCRs (Fig. S6). Moreover, the phylogenetic analyses of the MbdW, MbdX and MbdY enzymes

responsible for the modified β-oxidation within the 3-methylbenzoyl-CoA central pathway (Fig. 1) reveal that these proteins belong to the *Thauera/Geobacter/Magnetospirillum* cluster rather than to the *Azoarcus/Aromatoleum/Syntrophus* cluster of orthologous enzymes (Figs. S8-S10).

Therefore, all these phylogenetic data suggest that the evolutionary origin of the mbd pathway from *Azoarcus* sp. CIB differs to that of the classical bzd pathway from *Azoarcus/"Aromatoleum"* strains. In this sense, it is worth mentioning that the GC content of most *mbd* genes is lower than the average GC content of the whole *Azoarcus* sp. CIB genome (65.8%) (Valderrama *et al*., 2012) (Table 1). Moreover, the codon adaptation index of the *mbd* genes is also significantly lower than that of the majority of the genes from *Azoarcus* sp. CIB (>0.76) (Table 1). Such variations in GC content and codon adaptation index strongly suggest that the *mbd* gene cluster has been evolutionary recruited by *Azoarcus* sp. CIB from a different microorganism with a lower GC content through a horizontal gene transfer event.

Conclusions

The comparative genomic, biochemical and genetic data presented in this work revealed the existence of a new central pathway (*mbd* genes) for the anaerobic degradation of some aromatic compounds via the formation of 3-methylbenzoyl-CoA and methylated dienoyl-CoA products. A 3-methylbenzoate-dependent induction of a 3-methylbenzoate-CoA ligase (MbdA), a 3-methylbenzoyl-CoA reductase (MbdONQP) and the dienoyl-CoA hydratase (MbdW) that initiates the modified β-oxidation of the alicyclic compound has been shown. Moreover, the *mbd* cluster is proposed to encode the remaining enzymes involved in the 3-methylbenzoyl-CoA upper pathway, including the electron donor system needed for the reductase activity, and most of the lower

pathway enzymes. The existence of a devoted central pathway for the anaerobic degradation of 3-methylbenzoyl-CoA could explain why many strains use only toluene but not *m*-xylene as a hydrocarbon substrate (Widdel *et al*., 2010). This agrees also with the finding that most presently known anaerobic benzoate degraders do not grow using 3-methylbenzoate as carbon source (Song *et al.*, 2001; Carmona *et al.*, 2009).

Although the genetic organization of the *mbd* cluster has been elucidated and a specific regulatory gene (*mbdR*) has been proposed, further work needs to be done to characterize at the molecular level the regulatory system that controls the expression of this new anaerobic central pathway.

Experimental procedures

Bacterial strains, plasmids and growth conditions

The bacterial strains as well as the plasmids used in this work are listed in Table 2. *E. coli* strains were grown in LB medium (Sambrook and Russell, 2001) at 37ºC. When required, *E. coli* cells were grown anaerobically in M63 minimal medium (Miller, 1972) at 30 °C using the corresponding necessary nutritional supplements, 20 mM glycerol as carbon source, and 10 mM nitrate as terminal electron acceptor. *Azoarcus* sp. CIB strains were grown anaerobically in MC medium at 30 ºC using the appropriate carbon source and 10 mM nitrate as terminal electron acceptor as described previously (López-Barragán *et al*., 2004a). Whe *m*-xylene was used as sole carbon source, it was provided at 250 mM in 2,2,4,4,6,8,8-heptamethylnonan as an inert carrier phase. Where appropriate, antibiotics were added at the following concentrations: ampicillin (100 μg ml^{-1}), gentamycin (7.5 μg ml^{-1}), and kanamycin (50 μg ml^{-1}).

Molecular biology techniques

Standard molecular biology techniques were performed as previously described (Sambrook and Russel, 2001). Plasmid DNA was prepared with a High Pure plasmid isolation kit (Roche Applied Science). DNA fragments were purified with Gene-Clean Turbo (Q-BIOgene). Oligonucleotides were supplied by Sigma. The oligonucleotides employed for PCR amplification of the cloned fragments and other molecular biology techniques are summarized in Table S1. All cloned inserts and DNA fragments were confirmed by DNA sequencing with fluorescently labelled dideoxynucleotide terminators (Sanger *et al.*, 1977) and AmpliTaq FS DNA polymerase (Applied Biosystems) in an ABI Prism 377 automated DNA sequencer (Applied Biosystems). Transformation of *E. coli* cells was carried out by using the RbCl method or by electroporation (Gene Pulser; Bio-Rad) (Sambrook and Russel, 2001). The proteins were analyzed by SDS-PAGE and Coomassie-stained as described previously (Sambrook and Russel, 2001). The protein concentration was determined by the method of Bradford (Bradford, 1976) using bovine serum albumin as the standard.

Sequence data analyses

The nucleotide sequence of the *mbd* cluster from *Azoarcus* sp. CIB has been submitted to the GenBank™ with accession number HE801912. Nucleotide sequence analyses were done at the National Center for Biotechnology Information (NCBI) server (http://www.ncbi.nlm.nih.gov). Open reading frame searches were performed with the ORF Finder program at the NCBI server. Gene cluster search was performed at the KEGG server. The codon adaptation index (CAI) was determined at the CAIcaI server (Puigbò *et al.*, 2008) using the *Azoarcus* sp. CIB whole genome nucleotide sequence. The amino acid sequences of the open reading frames were compared with those present in databases using the TBLASTN algorithm (Altschul *et al.*, 1990) at the NCBI server (http://www.blast.ncbi.nlm.nih.gov). Pairwise and multiple protein sequence alignments

were made with the ClustalW program (Thompson *et al.*, 1994) at the EMBL-EBI server (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Phylogenetic analysis of the different proteins was carried out according to the Kimura two-parameter method (Kimura, 1980), and a tree was reconstructed using the neighbor-joining method (Saitou, 1987) of the PHYLIP program (Felsenstein, 1993) at the TreeTop-GeneBee server (http://www.genebee.msu.su/services/phtree_reduced.html) and represented using TreeView X 0.5.1 (Glasgow University).

RNA extraction and RT-PCR amplification

Azoarcus sp. CIB cells grown in MC medium harboring the appropriate carbon source were harvested at the mid-exponential phase of growth and stored at -80 °C. Pellets were thawed, and cells were lysed in TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) containing 5 mg ml^{-1} lysozyme. Total RNA was extracted using the RNeasy mini kit (Qiagen), including a DNase I treatment according to the manufacturer instructions (Ambion), precipitated with ethanol, washed, and resuspended in RNase-free water. The concentration and purity of the RNA samples were measured by using a ND1000 Spectrophotometer (Nanodrop Technologies) according to the manufacturer's protocols. Synthesis of total cDNA was carried out with 20 µl of reverse transcription reactions containing 400 ng of RNA, 0.5 mM concentrations of each dNTP, 200 U of SuperScript II reverse transcriptase (Invitrogen), and 5 µM concentrations of random hexamers as primers in the buffer recommended by the manufacturer. Samples were initially heated at 65 °C for 5 min, then incubated at 42 °C for 2 h, and the reactions were terminated by incubation at 70 °C for 15 min. In standard RT-PCR reactions, the cDNA was amplified with 1 U of AmpliTaq DNA polymerase (Biotools) and 0.5 µM concentrations of the corresponding primer pairs (Table S1). Control reactions in which reverse transcriptase was omitted from the reaction mixture ensured that DNA products

resulted from the amplification of cDNA rather than from DNA contamination. The *dnaE* gene encoding the α-subunit of DNA polymerase III was used to provide an internal control cDNA. The expression of the internal control was shown to be constant across all samples analysed.

Construction of Azoarcus sp. CIBdorf6, Azoarcus sp. CIBdorf5, Azoarcus sp. CIBdmbdY, Azoarcus sp. CIBdmbdO and Azoarcus sp. CIBdmbdA mutant strains

For insertional disruption of *orf6*, *orf5*, *mbdY*, *mbdO* and *mbdA* through single homologous recombination, an internal region of each gene was PCR-amplified with the primers indicated in Table S1. The obtained fragments were double digested with the appropriate restriction enzymes and cloned into double digested pK18*mob* vector generating the pK18orf6, pK18orf5, pK18mbdY, pK18mbdO and pK18mbdA recombinant plasmids (Table 2). The latter plasmids were transferred from *E. coli* S17-1λ*pir* (donor strain) to *Azoarcus* sp. CIB (recipient strain) by biparental filter mating (de Lorenzo and Timmis, 1994), and the exconjugant strains *Azoarcus* sp. CIBd*orf6*, *Azoarcus* sp. CIBd*orf5*, *Azoarcus* sp. CIBd*mbdY*, *Azoarcus* sp. CIBd*mbdO* and *Azoarcus* sp. CIBd*mbdA* were isolated aerobically on kanamycin-containing MC medium harbouring 10 mM glutarate as the sole carbon source for counterselection of donor cells. The mutant strains were analysed by PCR to confirm the disruption of the target gene.

Construction of a P_{B1}::lacZ fusion

The intergenic region between *mbdO* and *mbdB1* genes, the P_{B1} promoter region, was PCR-amplified using the PmbdB1KpnI/PmbdB1XbaI primers detailed in Table S1. The resulting 563 bp fragment was KpnI/XbaI double digested and cloned upstream of *lacZ* gene into the double digested pSJ3 promoter probe vector. The recombinant pSJ3P_{B1}

plasmid (Table 2) was KpnI/HindIII double digested, and the 3.7 kb fragment containing the P_{B1}*::lacZ* translational fusion was then cloned into the broad-host-range pIZ1016 cloning vector (Table 2). To this end, pIZ1016 was KpnI/HindIII double digested and its *Ptac* promoter and polylinker region were replaced by the P_{B1}*::lacZ* translational fusion, generating plasmid $pIZP_{B1}$ (Table 2).

Overproduction of MbdW-His_6 protein

The recombinant pETmbdW plasmid (Table 2) carries the *mbdW* gene with a His_6 tag coding sequence at its 3´-end, under control of the P_{T7} promoter which is recognised by the T7 phage RNA polymerase. The gene encoding this enzyme is present in monocopy in *E. coli* BL21 (DE3), and its transcription is controlled by the *Plac* promoter and the LacI repressor, making the system inducible by the addition of isopropyl-1-thio-β-*D*-galactopyranoside (IPTG). *E. coli* BL21 (DE3) (pETmbdW) cells were grown at 37 ºC in 100 ml kanamycin-containing LB medium until the culture reached an A_{600} of 0.5. Overexpression of the His-tagged protein was then induced during 5 h by the addition of 0.5 mM IPTG. Cells were harvested at 4 ºC, resuspended in 10 ml of 20 mM imidazole-containing working buffer (20 mM Tris-HCl, pH 7.9, 250 mM KCl, 10 % glycerol), and disrupted by sonication. Cell debris was removed by centrifugation at 20.000 g for 20 min at 4ºC, and the resulting supernatant was used as crude cell extract. The MbdW-His_6 protein was purified from the crude cell extract by a single-step Ni-chelating chromatography with a column (HisTrap HP 1ml Ni Sepharose High Performance, GE Healthcare) operated in an ÄKTA (GE Healthcare) FPLC system. The column was loaded with the crude extract, washed with six volumes of working buffer plus 20 mM imidazole, and then a linear gradient from 20 mM to 500 mM imidazole in working buffer was applied (flow rate of 1 ml min^{-1}) for 30 min. 2 ml fractions were harvested, and MbdW-His_6 protein eluted between 150 mM and 250 mM imidazole.

The purity of MbdW-His_6 protein was analyzed by 12.5 % SDS-PAGE. Fractions containing protein of the desired molecular mass (29 kDa) were dialyzed against working buffer plus 20 mM imidazole, concentrated to 2.1 mg protein ml^{-1} using Vivaspin 6 columns (10000 MWCO, Sartorius), and stored at -20 ºC.

Determination of the native molecular mass

The native molecular mass of purified MbdW-His_6 protein was determined by gel filtration chromatography using a FPLC Superdex 200 HR 10/30 (GE Healthcare) column operated in an ÄKTA (GE Healthcare) FPLC system. The buffer used for the gel filtration chromatography was the working buffer used for the purification of MbdW (see above) without imidazole. About 0.11 mg of purified MbdW-His_6 protein were applied at a flow rate of 0.5 ml min^{-1}. The molecular mass standards used were: blue dextran (2000 kDa, Sigma), apoferritin from horse spleen (443 kDa, Sigma), catalase from bovine liver (232 kDa, Sigma), albumin from bovine serum (67 and 134 kDa, Sigma), carbonic anhydrase from bovine erythrocytes (29 kDa, Serva), acetone (0.058 kDa, Grüssing).

Synthesis of CoA esters

Chemical synthesis of benzoyl-CoA and 3-methylbenzoyl-CoA was carried out as reported (Schachter and Taggart, 1953; Gross and Zenk, 1966). Cyclohex-1,5-diene-1-carbonyl-CoA and 6-hydroxycyclohex-1-ene-1-carbonyl-CoA were enzymatically synthesized from benzoyl-CoA by enriched benzoyl-CoA reductase from *T. aromatica* that contained traces of dienoyl-CoA hydratase, and the analogous methylated compounds were synthesized with cell extracts of *Azoarcus* sp. CIB, as described previously (Thiele *et al*., 2008). Isolation and tests for purity of the CoA esters were performed by preparative HPLC (Laempe *et al.*, 1999).

CoA ligase assays

CoA ligase activities were assayed using *E. coli* DH10B (pUCmbdA) cells (Table 2) that overproduce the MbdA protein. *E. coli* DH10B (pUCmbdA) cells were grown in ampicillin-containing LB medium for 12 h at 20 ºC. Once the culture reached an A_{600} of 0.2, the cells were harvested, resuspended and 100-fold concentrated in 100 mM Tris-HCl pH 8.5 buffer containing a mixture of protease inhibitors (Protease Inhibitor Cocktail Tablets Complete Mini EDTA-free, Roche), and disrupted by sonication. Cell debris was removed by centrifugation at 14.000 g for 20 min at 4ºC, and the resulting supernatant was used as crude cell extract for the assays. The CoA ligase activities were determined at 30 ºC through a direct spectrophotometric assay or via a coupled assay. For the direct assay, the reaction mixture (600 µl) contained 100 mM Tris-HCl (pH 8.5), 2mM dithiothreitol, 5 mM $MgCl_2$, 1 mM ATP, 0.4 mM CoA, 1 mM 3-methylbenzoate and different volumes of cell extract. The 3-methylbenzoyl-CoA formation was followed spectrophotometrically at 290 nm ($\varepsilon = 3.9\ mM^{-1}\ cm^{-1}$) as previously described (Niemetz *et al*., 1995). For the coupled assay, AMP formation was monitored by coupling the CoA ligase reaction to a myokinase, pyruvate kinase, and lactate dehydrogenase system and by spectrophotometrically measuring the rate of NADH oxidation at 365 nm (Ziegler *et al*., 1989). The reaction mixture (600 µl) contained 100 mM Tris-HCl (pH 8.5), 5 mM $MgCl_2$, 2 mM ATP, 1 mM CoA, 2 mM phosphoenolpyruvate, 0.5 mM aromatic compound, 0.5 mM NADH, 1.2 µl of a mixture of pyruvate kinase (1 U) and lactate dehydrogenase (1 U) from rabbit muscle (Roche), 0.6 µl (0.6 U) of myokinase from chicken muscle (Sigma-Aldrich), and different volumes of cell extract.

(Methyl)benzoyl-CoA reductase and (methyl)dienoyl-CoA hydratase assays

Extracts from substrate-adapted *Azoarcus* sp. CIB wild-type and mutant cells (~3 g wet weight per substrate condition) were anoxically prepared as previously described (Tschech and Fuchs, 1987). HPLC analysis of thioester intermediates of benzoyl- and 3-methylbenzoyl-CoA reductase and (methyl)cyclohex-1,5-diene-1-carbonyl-CoA hydratase activity were conducted as previously described (Möbitz and Boll, 2002; Peters *et al*., 2007). The technical variance of the assays is maximal 15%. (Methyl)cyclohex-1,5-diene-1-carbonyl-CoA hydratase activity was also measured in a continuous spectrophotometric assay at 320 nm as reported previously using the BamR hydratase from *S. aciditrophicus* as control (Laempe *et al*., 1998; Peters *et al*., 2007).

β-galactosidase assays

The β-galactosidase activities were measured with permeabilized cells when cultures reached mid-exponential phase as described by Miller (1972).

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Table 1. The *mbd* genes, their products, and related gene products.

Gene	D[a] (pb)	Gene Product aa/kDa	CAI/ %GC	Putative function of gene product	Related gene products				
					Name/Size (aa)	Function	Organism[b]	% ID	Accession no.
orf9	35	263/28.4	0.43/57.1	Enoyl-CoA hydratase	BH160DRAFT_2879 / 285	Putative enoyl-CoA hydratase/isomerase	*Burkholderia* sp. H160	72	EEA01841
orf8	12	336/36.1	0.39/56.9	Acyl-CoA dehydrogenase	Bcep18194_C7126 / 328	Putative acyl-CoA dehydrogenase	*Burkholderia* sp. 383	49	ABB06170
orf7	0	383/42.0	0.46/59.1	Acyl-CoA dehydrogenase	Bpet3290 / 383	Putative acyl-CoA dehydrogenase	*B. petrii* DSM 12804	70	CAP43632
orf6	481	437/46.3	0.44/58.1	Acetyl-CoA hydrolase	BH160DRAFT_2876 / 422	Putative acetyl-CoA hydrolase/transferase related to GCN5	*Burkholderia* sp. H160	55	EEA01838
orf5	158	378/41.0	0.48/59.1	Methylglutaryl-CoA dehydrogenase	MGR_2405 / 380	Putative butyryl-CoA dehydrogenase	*M. gryphiswaldense* MSR-1	73	CAM75442
korB2	40	341/37.0	0.68/64.3	2-oxoglutarate:ferredoxin oxidoreductase subunits	KorB2 / 341	Putative 2-oxoglutarate:ferredoxin oxidoreductase β subunit	*A. aromaticum* EbN1	85	Q5P471
korA2	202	635/68.1	0.69/64.9		KorA2 / 636	Putative 2-oxoglutarate:ferredoxin oxidoreductase α subunit	*A. aromaticum* EbN1	87	Q5P472
mbdM	634	80/8.5	0.43/50.8	Ferredoxin	Fdx / 81	Ferredoxin (two [4Fe-4S])	*T.aromatica* K172	61	CAA05141
mbdW	13	256/27.7	0.48/60.4	Dienoyl-CoA hydratase	Dch/ 258	Cyclohex-1,5-diene-1-carbonyl-CoA hydratase	*T. aromatica* K172	61	CAA12246
mbdY	24	378/40.9	0.66/62.5	Oxoacyl-CoA hydrolase	Oah / 377	6-ketocyclohex-1-ene-1-carbonyl-CoA hydrolase	*T. aromatica* K172	64	CAA12245
mbdX	85	355/38.1	0.61/63.5	Hydroxyacyl-CoA dehydrogenase	Had / 368	6-hydroxycyclohex-1-ene-1-carbonyl-CoA dehydrogenase	*T. aromatica* K172	65	CAA12244
orf4	12	287/31.6	0.58/59.6	Hydroxyacyl-CoA dehydrogenase	Hbd3 / 289	Putative 3-hydroxybutyryl-CoA dehydrogenase	*C. hydrogenoformans* Z-2901	51	ABB14715
orf3	10	256/27.7	0.62/61.0	Enoyl-CoA hydratase	Crt1 / 257	Putative enoyl-CoA hydratase	*C. hydrogenoformans* Z-2901	38	CAY33939
orf2	13	398/41.7	0.55/61.4	Thiolase	BH160DRAFT_2875 / 398	Putative thiolase	*Burkholderia* sp. H160	67	EEA01837
orf1	621	280/32.1	0.62/56.0	Unknown	Htur_4417 / 286	Putative amidohydrolase 2	*H. turkmenica* DSM 5511	44	ADB63228
mbdP	3	273/29.5	0.49/55.5	3-methylbenzoyl-CoA reductase subunits	BcrD / 282	Benzoyl-CoA reductase δ subunit	*T. aromatica* K172	33	O87877
mbdQ	21	269/28.4	0.51/58.4		BadF / 437	Benzoyl-CoA reductase α subunit	*R. palustris* CGA009	48	AAC23927
mbdN	20	388/44.1	0.49/52.7		BcrC / 386	Benzoyl-CoA reductase γ subunit	*T. aromatica* K172	34	CAA12247
mbdO	543	445/49.9	0.46/51.7		BadE / 436	Benzoyl-CoA reductase β subunit	*R. palustris* CGA009	34	AAC23926
mbdB1	104	397/42.4	0.58/60.4	ABC transporter subunits	ebA5303 / 395	Putative benzoate transporter, periplasmic subunit	*A. aromaticum* EbN1	68	Q5P0M6
mbdB2	1	290/31.3	0.51/57.5		ebA5304 / 288	Putative benzoate transporter, permesase	*A. aromaticum* EbN1	72	Q5P0M5
mbdB3	0	329/35.8	0.57/59.2		azo3048 / 326	Putative benzoate transporter, permesase	*Azoarcus* sp. BH72	58	CAL95665
mbdB4	0	259/27.4	0.52/60.4		ebA5307 / 253	Putative benzoate transporter, ATPase	*A. aromaticum* EbN1	66	Q5P0M3
mbdB5	231	238/25.9	0.52/59.5		ebA5309 / 238	Putative benzoate transporter, ATPase	*A. aromaticum* EbN1	69	Q5P0M2
mbdA	214	526/57.9	0.50/56.2	3-methylbenzoate-CoA ligase	BzdA / 533	Benzoate-CoA ligasa	*Azoarcus* sp. CIB	47	AAQ08820
mbdR	-	214/23.8	0.45/49.1	Transcriptional regulator	AcrR / 215	Transcriptional regulator (TetR family)	*E. coli* BL21 (DE3)	23	CAQ30937

[a] Distance of intergenic region to next gene.

[b] The full names of the organisms are: *Bordetella petrii* DSM 12804, *Magnetospirillum gryphiswaldense* MSR-1, "*Aromatoleum aromaticum*" EbN1, *Thauera aromatica* K172, *Carboxydothermus hydrogenoformans* Z-2901, *Haloterrigena turkmenica* DSM 5511, *Rhodopseudomonas palustris* CGA009, *Escherichia coli* BL21 (DE3).

Table 2. Bacterial strains and plasmids used in this work.

Strain or plasmid	Description	Reference
Strains		
E. coli		
DH10B	F´, *mcrA*, Δ(*mrr*, *hsdRMS-mcrBC*), ϕ80d*lac*ΔM15, Δ*lacX74*, *deoR*, *recA1*, *araD139*, Δ(*ara-leu*)*7697*, *galU*, *galK* λ-, *rpsL*, *endA1*, *nupG*	Life Technologies
BL21 (DE3)	F^-, *ompT*, $hsdS_B(r_B^-m_B^-)$, *gal*, *dcm*, λDE3	Sambrook and Russel, 2001
S17-1λpir	Tp^r, Sm^r, *recA*, *thi*, *hsdRM*+, RP4::2-Tc::Mu::Km, Tn*7*, λ*pir* phage lysogen	de Lorenzo and Timmis, 1994
Azoarcus sp. CIB[a]		
Azoarcus sp. CIB	Wild-type strain. 3MBz (+) / *m*-Xyl (+)/ Bz (+) / Tol(+)	López-Barragán *et al.*,2004a
Azoarcus sp.CIBd*mbdO*	CIB mutant strain with a disruption of the *mbdO* gene. 3MBz (-) / *m*-Xyl (-)/ Bz (+) / Tol(+)	This work
Azoarcus sp.CIBd*mbdY*	CIB mutant strain with a disruption of the *mbdY* gene. 3MBz (-) / *m*-Xyl (-)/ Bz (+) / Tol(+)	This work
Azoarcus sp.CIBd*mbdA*	CIB mutant strain with a disruption of the *mbdA* gene. 3MBz (-) / *m*-Xyl (+)/ Bz (+) / Tol(+)	This work
Azoarcus sp. CIBd*orf5*	CIB mutant strain with a disruption of the *orf5* gene. 3MBz (-) / *m*-Xyl (-)/ Bz (+) / Tol(+)	This work
Azoarcus sp.CIBd*orf6*	CIB mutant strain with a disruption of the *orf6* gene. 3MBz (-) / *m*-Xyl (-)/ Bz (+) / Tol(+)	This work
Azoarcus sp. CIBd*bzdN*	CIB mutant strain with a disruption of the *bzdN* gene. 3MBz (+) / *m*-Xyl (+)/ Bz (-) / Tol(-)	López-Barragán *et al.*,2004a
Azoarcus sp. CIBd*acpR*	CIB mutant strain with a disruption of the *acpR* gene. 3MBz (-) / *m*-Xyl (-)/ Bz (-) / Tol(-)	Durante-Rodríguez *et al.*,2006
Plasmids		
pK18*mob*	Km^r, *ori*ColE1, Mob^+, *lacZ*α, used for directed insertional disruption	Schäfer *et al.*, 1994
pK18mbdO	Km^r, pK18*mob* containing a 787 bp HindIII/XbaI *mbdO* internal fragment	This work
pK18mbdY	Km^r, pK18*mob* containing a 709 bp HindIII/XbaI *mbdY* internal fragment	This work
pK18mbdA	Km^r, pK18*mob* containing a 736 bp HindIII/XbaI *mbdA* internal fragment	This work
pK18orf5	Km^r, pK18*mob* containing a 585 bp HindIII/XbaI *orf5* internal fragment	This work
pK18orf6	Km^r, pK18*mob* containing a 723 bp HindIII/XbaI *orf6* internal fragment	This work
pUC19	Ap^r, *ori*ColE1, *lacZ*α, high-copy number cloning vector	Sambrook and Russel, 2001
pUCmbdA	Ap^r, pUC19 expressing the *mbdA* gene under control of *Plac*	This work
pET-29a(+)	Km^r, *ori*ColE1, P_{T7} , cloning and overexpression vector	Novagen
pETmbdW	Km^r, pET-29a (+) expressing *mbdW*-His_6 gene under P_{T7}	This work
pSJ3	Ap^r, *ori*ColE1, ´*lacZ* promoter probe vector	Ferrández *et al.*, 1998
$pSJ3P_{B1}$	Ap^r, pSJ3 derivative carrying the P_{B1}::*lacZ* fusion	This work
pIZ1016	Gm^r, *ori*pBBR1, Mob^+, *lacZα*, *Ptac*/$lacI^q$, broad-host-range cloning and expression vector	Moreno-Ruiz *et al.*, 2003
$pIZP_{B1}$	Gm^r, pIZ1016 derivative expressing the P_{B1}::*lacZ* fusion	This work
pIZ-FNR*	Gm^r, pIZ1016 derivative containing the *fnr** gene under *Ptac*	Durante-Rodríguez *et al.*, 2006

a. For *Azoarcus* sp. CIB strains the ability (+) or inability (-) to grow using 3-methylbenzoate (3MBz), *m*-xylene (*m*-Xyl), benzoate (Bz), or toluene (Tol) as sole carbon source is indicated.

Table 3. (3-methyl)benzoyl-CoA reductase activities in extracts from *Azoarcus* sp. CIB, *Azoarcus* sp. CIBd*mbdO* and *Azoarcus* sp. CIBd*bzdN* strains.

Extracts from cells grown on[a]	Substrate in *in vitro* assay[b]	Reductase activity [nmol min^{-1} mg $protein^{-1}$]		
		***Azoarcus* sp. CIB**	***Azoarcus* sp. CIBd*mbdO***	***Azoarcus* sp. CIBd*bzdN***
Bz	Bz-CoA	32.3	49.8	nd
	3MBz-CoA	16.5	12.6	nd
3MBz	Bz-CoA	26.8	nd[c]	34.3
	3MBz-CoA	11.3	nd	10.6
Glutarate	Bz-CoA	< 0.1	nd	nd
	3MBz-CoA	< 0.1	nd	nd
Glutarate/3MBz	Bz-CoA	16.5	< 0.1	nd
	3MBz-CoA	7.5	< 0.1	nd

a. Bz, benzoate; 3MBz, 3-methylbenzoate.

b. Bz-CoA, benzoyl-CoA; 3MBz-CoA, 3-methylbenzoyl-CoA.

c. nd, not determined.

Figure legends

Fig. 1. The 3-methylbenzoate anaerobic degradation pathway in *Azoarcus* sp. CIB.

A. Scheme of the *mbd* gene cluster involved in the anaerobic catabolism of 3-methylbenzoate in *Azoarcus* sp. CIB. Genes are represented by arrows and their predicted function (Table 1) is annotated as follows: grey, regulatory gene; stippling, gene encoding the 3-methylbenzoate-CoA ligase; black, genes encoding the four subunits of the 3-methylbenzoyl-CoA reductase; wavy, genes encoding the electron donor system of the 3-methylbenzoyl-CoA reductase; vertical stripes, genes encoding the modified β-oxidation reaction sequence; horizontal stripes, genes encoding a 3-methylbenzoate ABC-type transport system; white, genes involved in the 3-methylbenzoyl-CoA lower pathway (and some genes of unknown function). The cross hatching thin arrows represent the *tdiSR* and *bss* genes involved in the peripheral toluene/*m*-xylene degradation pathway (B. Blázquez, unpublished).

B. Scheme of 3-methylbenzoate activation and the proposed 3-methylbenzoyl-CoA upper degradation pathway (dearomatization and modified β-oxidation). The enzymes are indicated following the color code indicated in (A). Enzyme names and predicted functions are provided in Table 1. Since the reduction of 3-methylbenzoyl-CoA can generate two different reaction products, i.e., 3a or 3b, the next intermediates in the modified β-oxidation can be also the a or b forms, i.e., 4a-6a or 4b-6b, respectively. The compounds are as follows: 1, 3-methylbenzoate; 2, 3-methylbenzoyl-CoA; 3a, 3-methyl-cyclohex-1,5-diene-1-carbonyl-CoA; 3b, 5-methyl-cyclohex-1,5-diene-1-carbonyl-CoA; 4a, 5-methyl-6-hydroxycyclohex-1-ene-1-carbonyl-CoA; 4b, 3-methyl-6-hydroxycyclohex-1-ene-1-carbonyl-CoA; 5a, 5-methyl-6-ketocyclohex-1-ene-1-carbonyl-CoA; 5b, 3-methyl-6-ketocyclohex-1-ene-1-carbonyl-CoA; 6a, 3-hydroxy-6-methyl-pimelyl-CoA; 6b, 3-hydroxy-4-methyl-pimelyl-CoA.

Fig. 2. Substrate specificity of 3-methylbenzoate CoA ligase (MbdA) enzyme. The CoA ligase activity values were determined by using the coupled assay described in *Experimental procedures.* Activity values are represented as a percentage of the activity with 3-methylbenzoate as substrate (0.11 μmol min^{-1} mg $protein^{-1}$). The compounds tested (at a concentration of 0.5 mM) are: 3-methylbenzoate (3MBz); benzoate (Bz); 2-methylbenzoate (2MBz); 4-methylbenzoate (4MBz); 2-fluorobenzoate (2FBz); 3-fluorobenzoate (3FBz); 4-fluorobenzoate (4-FBz); 2-chlorobenzoate (2CBz); 3-chlorobenzoate (3CBz); 4-chlorobenzoate (4-CBz); 3-hydroxybenzoate (3HBz); phenylpropionate (PP); 3-hydroxyphenylpropionate (3HPP); phenylacetate (PA). Values are the mean of three different experiments. Error bars indicate standard deviation.

Fig. 3. HPLC analyses of the 3-methylbenzoyl-CoA and benzoyl-CoA reductase activity in *Azoarcus* sp. CIB extracts from 3-methylbenzoate-grown cells. Each panel shows the reaction assay at the beginning (upper) and after 5 min incubation (lower).

A. Time-dependent reduction of 3-methylbenzoyl-CoA (0.2 mM).

B. Time-dependent reduction of benzoyl-CoA (0.2 mM)..

The compounds shown are: 2, 3-methylbenzoyl-CoA; 2´, benzoyl-CoA; 3, methyl-cyclohex-1,5-diene-1-carbonyl-CoA (methyldienoyl-CoA); 3´, cyclohex-1,5-diene-1-carbonyl-CoA (dienoyl-CoA). Additional HPLC peaks represent further intermediates of methyldienoyl-CoA and dienoyl-CoA degradation.

Fig. 4. HPLC analyses of the (methyl)-6-hydroxymonoenoyl-CoA dehydratase activity of purified MbdW protein. Each panel shows the reaction assay at the beginning (upper) and after 1 min incubation (lower) with purified MbdW-His_6 protein (0.7 μM).

A. Time-dependent dehydration of methyl-6-hydroxycyclohex-1-ene-1-carbonyl-CoA (methyl-6-hydroxymonoenoyl-CoA) (0.5 mM).

B. Time-dependent dehydration of 6-hydroxycyclohex-1-ene-1-carbonyl-CoA (6-hydroxymonoenoyl-CoA) (0.5 mM).

The main peaks of the chromatograms are indicated: 4, methyl-6-hydroxymonoenoyl-CoA; 3, methyl-dienoyl-CoA; 4´, 6-hydroxymonoenoyl-CoA; 3´, dienoyl-CoA.

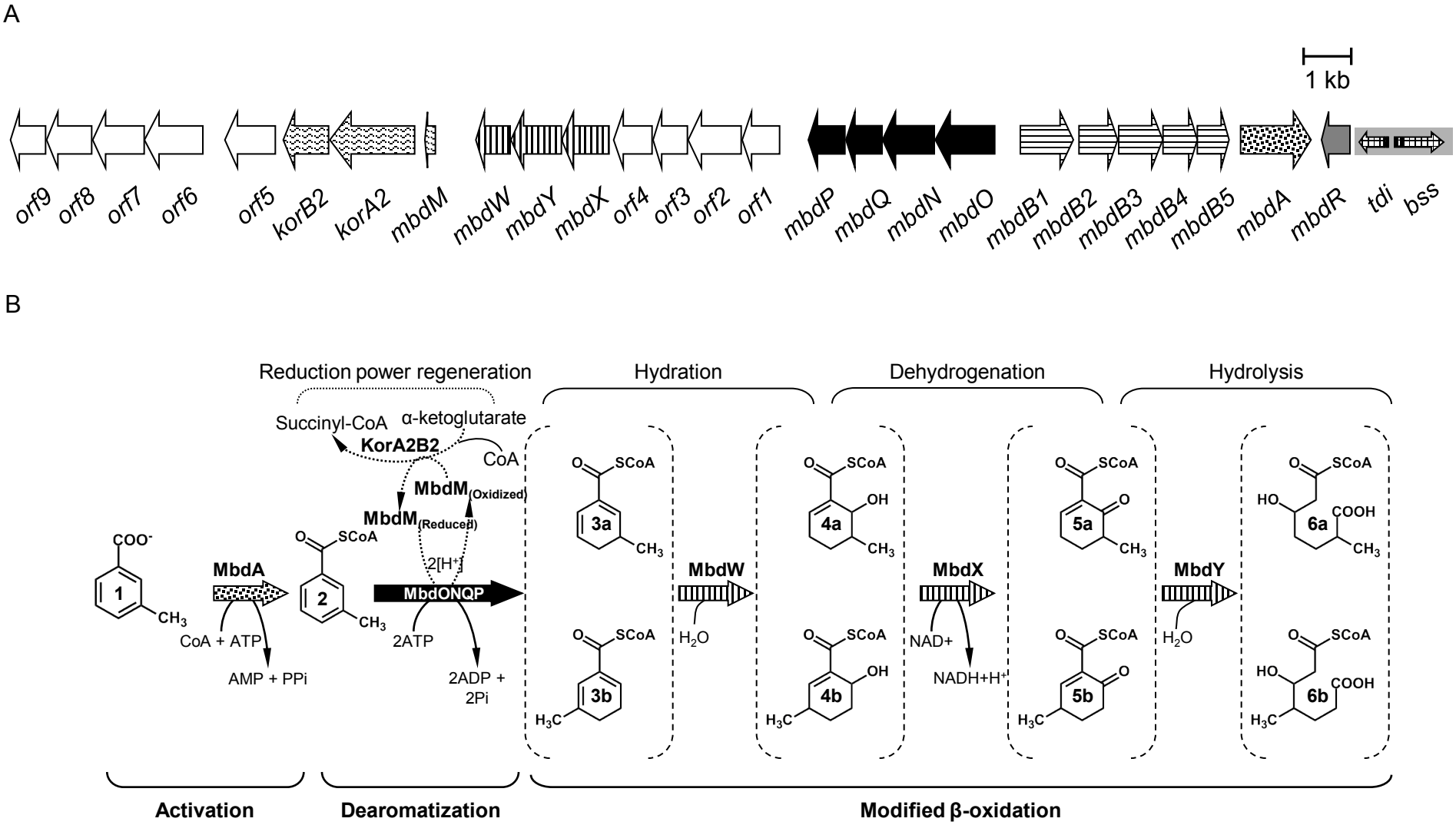
Fig. 5. Transcriptional organization of the *mbd* cluster.

A. Schematic representation of the *mbd* cluster from *Azoarcus* sp. CIB. The catabolic, transport and regulatory genes are indicated by black, stripped and grey arrows, respectively. The P_O, P_{B1} and P_{3R} promoters are represented by bent arrows. The intergenic regions whose expression was analyzed by RT-PCR are shown (a to i) and their size (in bp) is indicated in brackets.

B. Agarose gel electrophoresis of RT-PCR products. RT-PCRs with *Azoarcus* sp. CIB cells grown under denitrifying conditions on 3-methylbenzoate (lanes 3M) or benzoate (lanes B) were performed as described in *Experimental procedures* with the primer pairs (Table S1) that amplify each of the intergenic regions indicated in (A). Lanes M, molecular size markers (HaeIII-digested ΦX174 DNA). Numbers on the left represent the sizes of the markers (in bp).

Fig. 6. Activity of the P_O, P_{B1} and P_{3R} promoters in wild-type *Azoarcus* sp. CIB and the *Azoarcus* sp. CIBd*acpR* mutant strain. Agarose gel electrophoresis of RT-PCR products obtained from P_O, P_{B1} and P_{3R} promoters. Total RNA was extracted from *Azoarcus* sp. CIB (wt) and *Azoarcus* sp. CIBd*acpR* (acpR) cells grown under denitrifying conditions using 0.2% alanine as carbon source (lanes A) or 0.2% alanine and 3 mM 3-methylbenzoate as inducer (lanes 3M). The primer pairs used to amplify the *mbdO* (P_O), *mbdB1* (P_{B1}) and *mbdR* (P_{3R}) gene fragments as described in *Experimental procedures* are detailed in Table S1. Lanes M, molecular size markers (HaeIII-digested ΦX174 DNA). Numbers on the left represent the sizes of the markers (in bp).

Fig. 7. Carbon catabolite repression of 3-methylbenzoate degradation in *Azoarcus* sp. CIB. *Azoarcus* sp. CIB cells containing plasmid $pIZP_{B1}$ (harbours the P_{B1}*::lacZ* fusion) were grown anaerobically in minimal medium using different carbon sources, i.e., 3mM benzoate (Bz), 0.2% alanine (Ala), 5 mM glutarate (Glt), 0.4% casamino acids (Caa), 0.2% pyruvate (Pyr), 3 mM cyclohexanecarboxylate (Chc), 3mM 3-methylbenzoate (3MBz). The bars show the β-galactosidase activity (in Miller units) of the cell cultures determined as indicated under *Experimental procedures*. White bars represent the activity from cultures grown in each of the different carbon sources. Stripped bars show the activity from cultures grown in mixtures of the cognate carbon source plus 3 mM 3-methylbenzoate. Values are the mean of three independent experiments. Error bars indicate standard deviation.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mbdR
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bss
B
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Hydration
Dehydrogenation
Hydrolysis
Succinyl-CoA
α-ketoglutarate
KorA2B2
CoA
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MbdM(Reduced)
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MbdONQP
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MbdY
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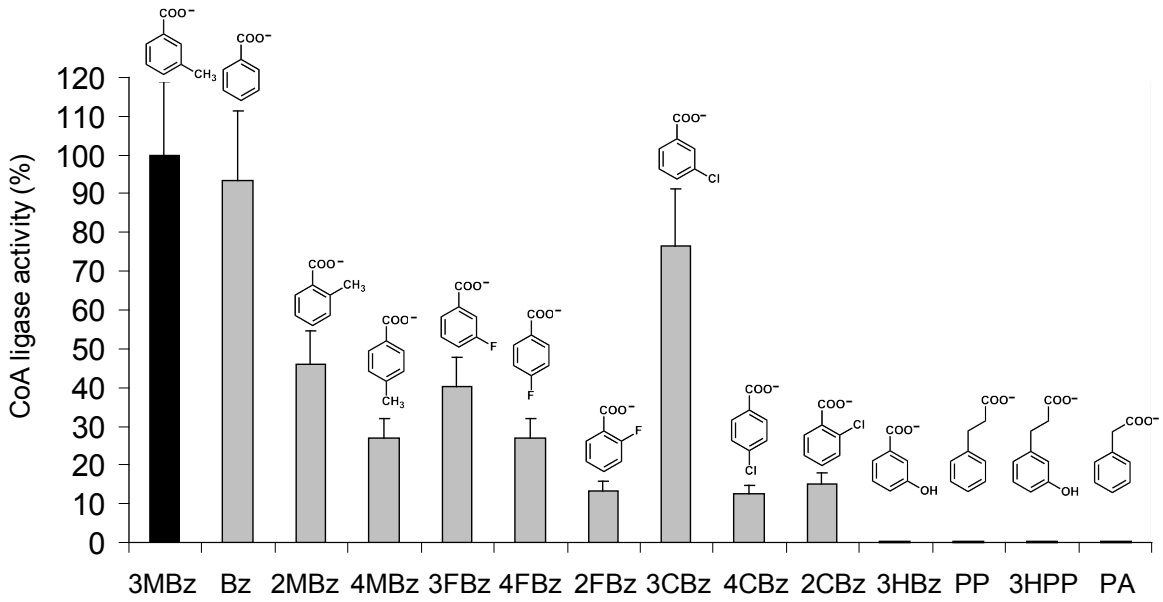
NAD+
NADH+H+
Activation
Dearomatization
Modified β-oxidation

Fig. 1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30
20
10
0
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Bz
2MBz
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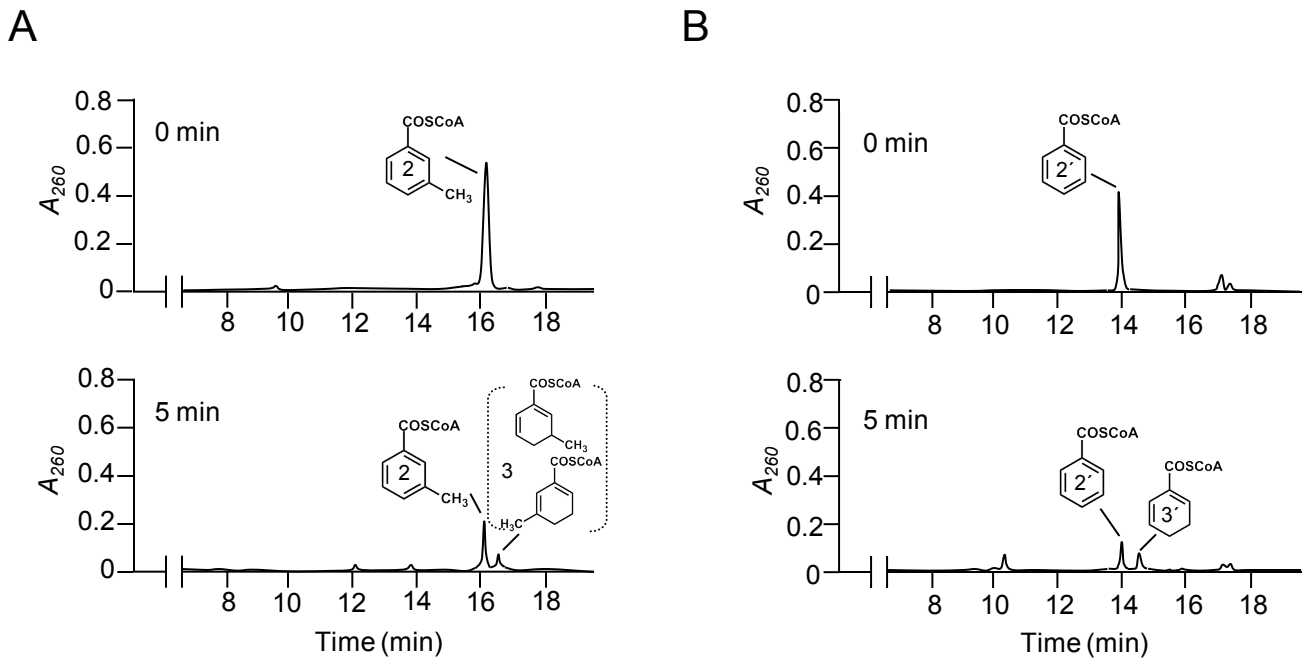
3FBz
4FBz
2FBz
3CBz
4CBz
2CBz
3HBz
PP
3HPP
PA

Fig. 2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B
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0.4
0.2
0
8
10
12
14
16
18
Time (min)
COSCoA
2
3
2´
3´
CH_3
H_3C

Fig. 3

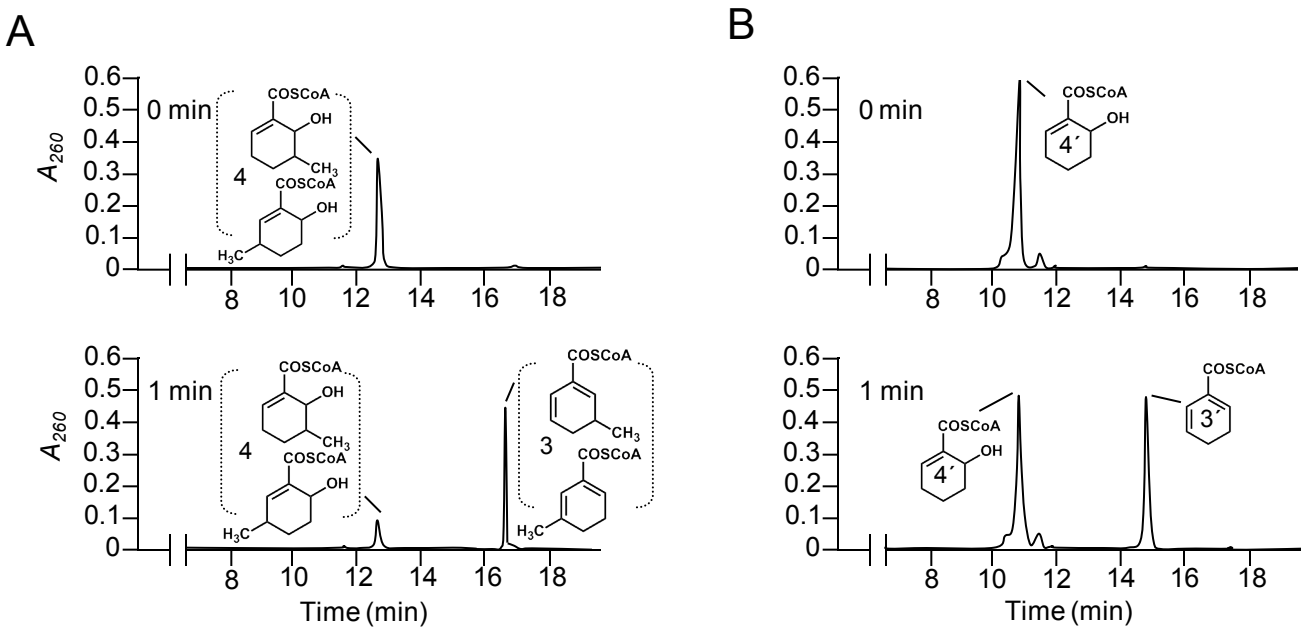
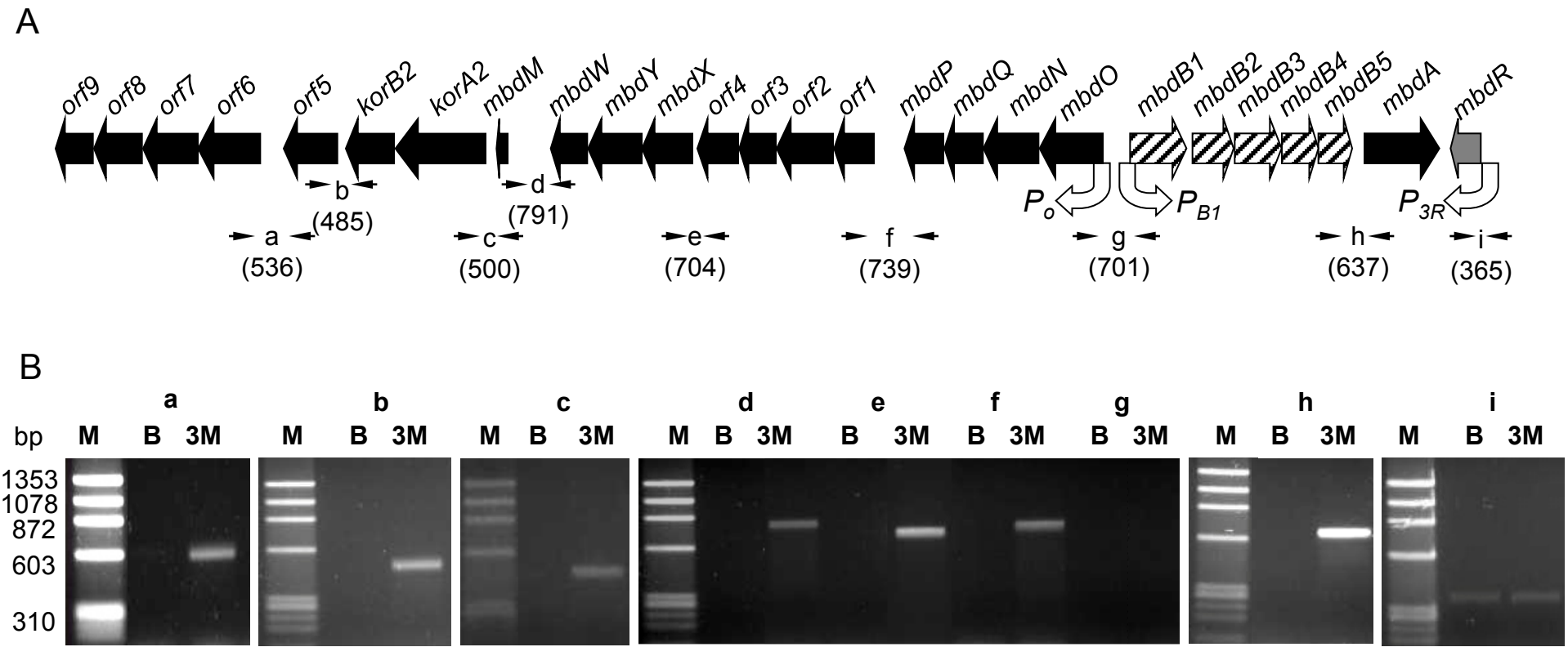


Fig. 4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orf2
orf1
mbdP
mbdQ
mbdN
mbdO
mbdB1
mbdB2
mbdB3
mbdB4
mbdB5
mbdA
mbdR
P_o
P_{B1}
P_{3R}
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b (485)
c (500)
d (791)
e (704)
f (739)
g (701)
h (637)
i (365)
B
a
b
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bp
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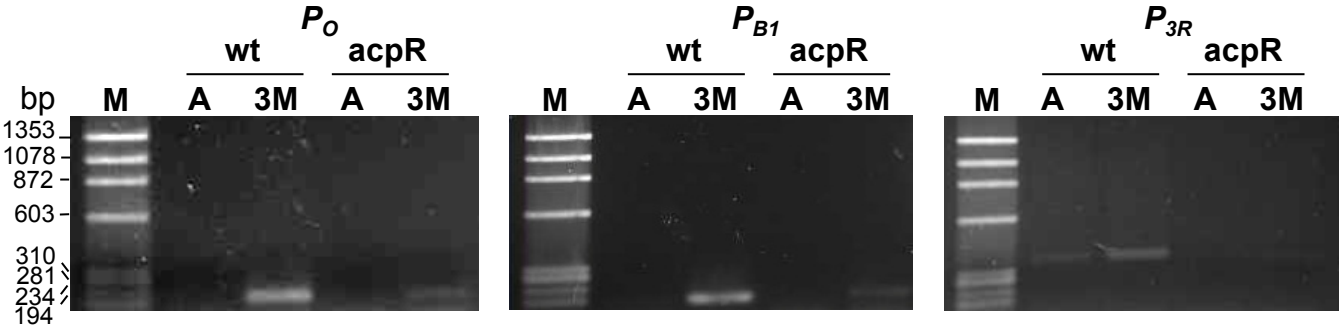
603
310

Fig. 5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bp
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wt
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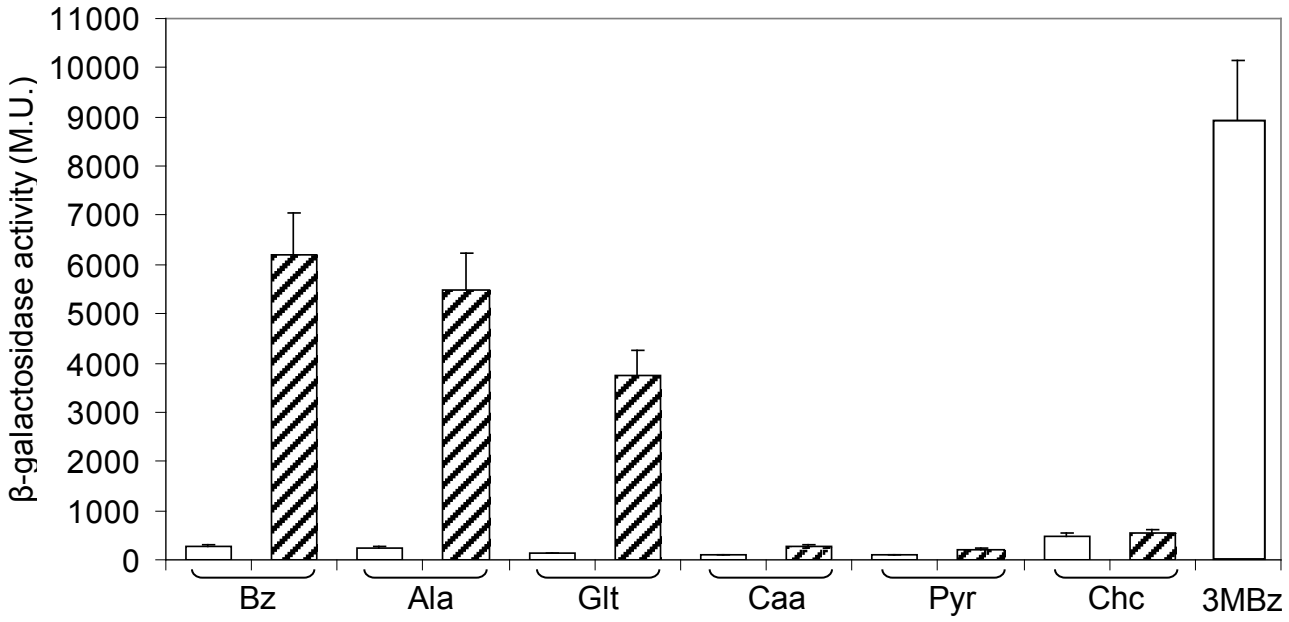
M
A
3M
A
3M

Fig. 6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7000
6000
5000
4000
3000
2000
1000
0
Bz
Ala
Glt
Caa
Pyr
Chc
3MBz

Fig. 7