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MicroCommentary

Iron-reducing bacteria unravel novel strategies for the anaerobic catabolism of aromatic compounds

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Summary

Although the aerobic degradation of aromatic compounds has been extensively studied in many microorganisms, the anaerobic mineralization of the aromatic ring is a more recently discovered microbial capacity on which very little information is available from facultative anaerobic bacteria. In this issue of *Molecular Microbiology*, Wischgoll and colleagues use proteomic and reverse-transcription polymerase chain reaction (PCR) approaches to identify for the first time the gene clusters involved in the central pathway for the catabolism of aromatic compounds in *Geobacter metallireducens*, a strictly anaerobic iron-reducing bacterium. This work highlights that the major difference in anaerobic benzoate metabolism of facultative and strictly anaerobic bacteria is the reductive process for dearomatization of benzoyl-CoA. The authors propose that a new type of benzoyl-CoA reductase, comprising molybdenum- and selenocysteine-containing proteins, is present in strictly anaerobic bacteria. This work paves the way to fundamental studies on the biochemistry and regulation of this new reductive process and provides the first genetic clues on the anaerobic catabolism of benzoate by strict anaerobes.

After carbohydrates, aromatic compounds, found as lignin monomers, flavonoids, quinones, aromatic amino acids, or main constituents of fossil fuels, are the most widely distributed class of organic compounds in nature. Moreover, a significant number of xenobiotics (e.g. polychlorinated biphenyls and dioxins, nitroaromatics, etc.) belong to this family of compounds. The thermodynamic stability of the benzene ring increases its persistence in the environment. This fact, together with the massive release of aromatics into the environment by man, means that many of such compounds, e.g. benzene, toluene, xylenes, ethylbenzene and phenol, are major pollutants. Due to the low chemical reactivity of aromatic compounds, their biodegradation requires unusual biochemical reactions, some of which are of great biotechnological potential. The catabolism of aromatic compounds, mainly carried out by microorganisms, involves a wide variety of peripheral pathways that channel structurally diverse substrates into a limited number of common intermediates that are further processed by a few central pathways to the central metabolism of the cell (Harwood *et al.*, 1999; Pieper *et al.*, 2004). There are two major strategies to degrade aromatic compounds, depending on the presence or absence of oxygen. In the aerobic catabolism of aromatics, oxygen is not only the final electron acceptor but also a co-substrate for two key processes such as the hydroxylation and oxygenolytic ring cleavage of the benzene ring. In contrast, the anaerobic catabolism of aromatic compounds uses a completely different strategy, based on reductive reactions, to attack the aromatic ring (Harwood *et al.,* 1999; Gibson and Harwood, 2002). As many ecosystems are often anoxic (e.g. aquifers, aquatic sediments and submerged soils), the anaerobic catabolism of aromatic compounds by microorganisms becomes crucial in the biogeochemical cycles and in the sustainable development of the biosphere (Widdel and Rabus, 2001; Lovley, 2003). Although the aerobic degradation of aromatic compounds has been extensively studied in many microorganisms, the anaerobic processes leading to mineralization of the aromatic ring are a more recently discovered microbial capacity and much less information is available, which makes its study of great interest from both the basic and the applied point of view. Although some peripheral pathways in anaerobic degradation of aromatic compounds lead to central intermediates such as resorcinol, phloroglucinol and hydroxyhydroquinone, most of the aromatic compounds are channelled to the common benzoyl-coenzymeA (CoA) intermediate (Harwood *et al.,* 1999; Gibson and Harwood, 2002). The anaerobic catabolism of the model compound benzoate via benzoyl-CoA has been studied at the molecular level in only a few facultative

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anaerobes, i.e. in the denitrifying bacteria *Thauera aromatica* and *Azoarcus* sp. (Breese *et al.,* 1998; Harwood *et al.,* 1999; Barragán *et al.,* 2004a; Rabus *et al.,* 2005) (β proteobacteria), and *Magnetospirillum magnetotacticum* (α proteobacteria) (Barragán *et al.,* 2004b), and in the photosynthetic bacterium *Rhodopseudomonas palustris* (α proteobacteria) (Egland *et al.,* 1997). In these bacteria, benzoate degradation starts with the activation to benzoyl-CoA via an ATP-dependent benzoate-CoA ligase that releases AMP and PPi (Fig. 1A). The second reaction step, reduction of the benzene ring to a non-aromatic (alicyclic) compound, is an energy-demanding, oxygen-sensitive process that is carried out by a benzoyl-CoA reductase (BCR). Whereas benzoyl-CoA is reduced to a cyclohexadienecarbonyl-CoA intermediate in *T. aromatica* and *Azoarcus* sp., a four-electron reduction, generating cyclohex-1-ene-carbonyl-CoA, was reported in *R. palustris* (Fig. 1A). Two slightly different modified β-oxidation sets, *Rhodopseudomonas*-type and *Thauera*-type, involving the introduction of a hydroxyl group, a dehydrogenation reaction, and a hydrolytic ring fission, then lead to the formation of an aliphatic C7-dicarboxyl-CoA, i.e. pimelyl-CoA in *R. palustris* and 3-hydroxypimelyl-CoA in *T. aromatica*, *Azoarcus* sp. and *M. magnetotacticum* (Fig. 1A). Further degradation of the dicarboxylic acid via β-oxidation reactions and a decarboxylation step (lower pathway) yields three molecules of acetyl-CoA and one CO_2. In denitrifying bacteria, acetyl-CoA is fully oxidized to CO_2 in a modified tricarboxylic acid-cycle with nitrate acting as final electron acceptor in an anaerobic respiratory chain. In photosynthetic bacteria, energy is derived from light and acetyl-CoA is used in biosynthetic reactions (Harwood *et al.,* 1999; Gibson and Harwood, 2002). Most of the genes involved in the anaerobic catabolism of benzoate to the aliphatic dicarboxyl-CoA in *R. palustris* (*bad* genes), *T. aromatica* and *M. magnetotacticum* (*bcr* genes), and *Azoarcus* sp. (*bzd* genes) are arranged in a single cluster (Fig. 1B).

Despite that fact that knowledge of the anaerobic catabolism of aromatic compounds by facultative anaerobes has increased significantly during the last decade, little is known about aromatic catabolism by strict anaerobes. Strictly anaerobic bacteria that use aromatic compounds as sole energy and carbon source have been isolated. They have anaerobic respiratory chains with Fe(III) or sulphate as terminal electron acceptors or are fermentative bacteria that often occur in syntrophic association with a methanogen (Schink *et al*., 2000; Elshahed *et al*., 2001; Widdel and Rabus, 2001; Peters *et al*., 2004). As indicated above, reduction of the benzene ring is an endergonic reaction that requires a rather high-energy input in the form of ATP. Whereas the free-energy change of complete benzoate oxidation in nitrate-reducing bacteria provides the required high-energy input for substrate-activation and dearomatization, the energy balance in strict anaerobes is less favourable and therefore an intriguing question arises: how can electron transfer to the aromatic ring be accomplished without a stoichiometric coupling to ATP hydrolysis in strict anaerobes? In this issue of *Molecular Microbiology*, Wischgoll and colleagues identify the gene clusters involved in benzoate catabolism in *Geobacter metallireducens*, an iron-reducing bacterium that is able to degrade aromatic compounds, and provide some answers to the question mentioned above.

Microbial Fe(III) reduction is considered to play an important role in a variety of processes of environmental concern. The largest known group of ferric iron reducers is the *Geobacteraceae* family in the δ subclass of proteobacteria. Within this family, some species use aromatic compounds as sole carbon and energy sources, as is the case of *G. grbiciae* TACP-5 (uses benzoate and toluene) and *G. hydrogenophilus* H-2 (uses benzoate) (Coates *et al*., 2001). However, the only species of *Geobacter* that is able to mineralize aromatic compounds and whose genome is known is *G. metallireducens*, a rod-shaped, non-motile, non-spore-forming, strictly anaerobic chemoorganotrophic bacterium that completely oxidizes toluene, benzoate, benzaldehyde, benzylalcohol, *p*-hydroxybenzoate, *p*-hydroxybenzaldehyde, *p*-hydroxybenzylalcohol, phenol or *p*-cresol to carbon dioxide with Fe(III) as the electron acceptor (Lovley *et al*., 1993). Beyond the opportunities for bioremediation, interest in *Geobacter* spp. lies in biotechnological efforts to capture energy from the catabolism of organic waste with energy-harvesting electrodes and in the ability of these bacteria to precipitate soluble metals, such as uranium, as products of electron transport (Lovley, 2003).

By using a proteomic approach, Wischgoll *et al*. (2005) identified 14 unknown proteins that are specifically induced when *G. metallireducens* cells are grown on benzoate. These results enabled the identification of the corresponding genes in the genome. Notably, these genes were not present in the genome of *G. sulforreducens*, which cannot utilize aromatic compounds. These genes are organized in two clusters, IA/B and II, comprising 44 open reading frames. Reverse-transcription experiments confirmed that expression of these genes is specifically induced in the presence of benzoate and indicated that adjacent genes in the same orientation might form a transcriptional unit. Clusters IA and II might encode mostly enzymes involved in the benzoyl-CoA central pathway and, for this reason, the genes are termed *bamA-bamY* (benzoic acid metabolism). On the other hand, cluster IB contains genes considered to be involved in the lower pathway of anaerobic benzoate degradation, together with the *bamM* gene product of cluster IA. Heterologous expression of the *bamY* gene, located at one end of

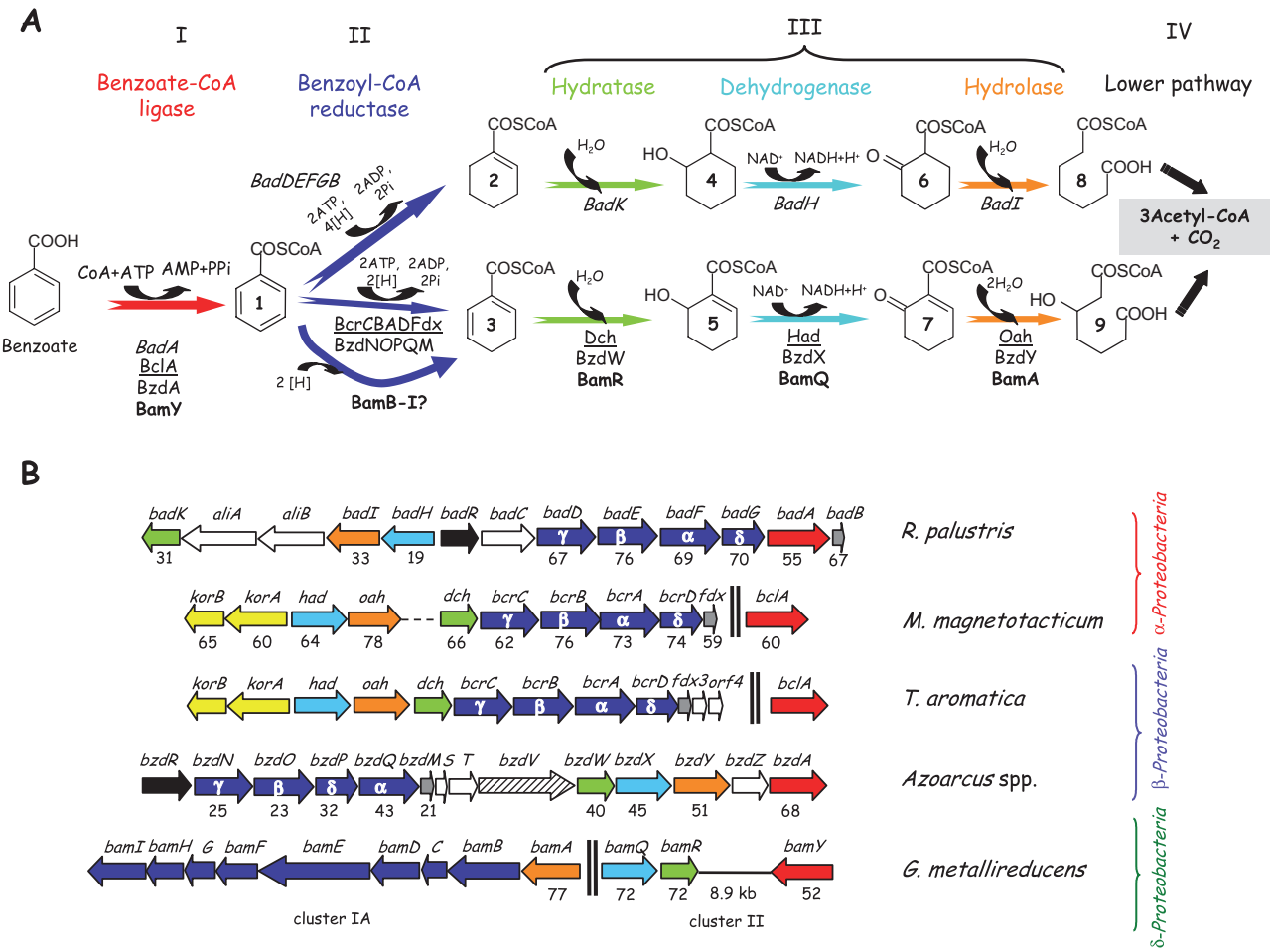

Fig. 1. Biochemistry and genetics of the anaerobic catabolism of benzoate in bacteria.
A. Biochemistry of the benzoate anaerobic pathway. The metabolites are: benzoyl-CoA (1), cyclohex-1-ene-1-carbonyl-CoA (2), cyclohex-1,5-diene 1-carbonyl-CoA (3), 2-hydroxy-cyclohexane-1-carbonyl-CoA (4), 6-hydroxycyclohex-1-ene-1-carbonyl-CoA (5), 2-ketocyclohexane-1-carbonyl-CoA (6), 6-ketocyclohex-1-ene-1-carbonyl-CoA (7), pimelyl-CoA (8) and 3-hydroxypimelyl-CoA (9). The *R. palustris* enzymes (Bad) are indicated in italics, *T. aromatica* and *M. magnetotacticum* enzymes (Bcr, Dch, Had, Oah) are underlined, *Azoarcus* sp. enzymes (Bzd) are indicated as plain text and *G. metallireducens* enzymes (Bam) are indicated in bold. The different reaction steps are indicated at the top of the figure: step I, activation of benzoate to benzoyl-CoA by benzoate-CoA ligase (red arrow); step II, dearomatization of benzoyl-CoA by benzoyl-CoA reductase (dark blue arrows); step III, modified β-oxidation reactions carried out by a hydratase (green arrows), dehydrogenase (light blue arrows) and ring cleaving hydrolase (orange arrows); and step IV, β-oxidation and decarboxylation reactions (lower pathway) of the C_7-dicarboxyl-CoA aliphatic intermediate (black arrow). Although all pathway intermediates shown in the figure have been demonstrated in *R. palustris* and *T. aromatica*, step III is proposed in *Azoarcus* sp., and steps II and III are proposed in *M. magnetotacticum* and *G. metallireducens*.
B. Organization of the gene clusters involved in the anaerobic catabolism of benzoate in *R. palustris* (GenBank Accession No. U75363), *M. magnetotacticum* MS-1 (GenBank Accession No. NZ_AAAP00000000), *T. aromatica* (GenBank Accession No. AJ224959), *Azoarcus* sp. (GenBank Accession No. AF515816, CR555306 and AJ428529 for *Azoarcus* sp. CIB, *Azoarcus* sp. EbN1 and *A. evansii* respectively) and *G. metallireducens* (GenBank Accession No. AAAS00000000). The predicted functions of some gene products in *Azoarcus* sp., *M. magnetotacticum* and *G. metallireducens* are based on biochemical information reported for the equivalent products in *R. palustris* and *T. aromatica*. Genes are represented by arrows: red, genes encoding benzoate-CoA ligases; dark blue, genes encoding the subunits of the benzoyl-CoA reductase (the α, β, γ, and δ subunits of the benzoyl-CoA reductase from facultative anaerobes are indicated); grey, genes encoding ferredoxins associated to the benzoyl-CoA reductases; green, genes encoding enoyl-CoA hydratases; light blue, genes encoding enoyl-CoA dehydrogenases; orange, genes encoding ring-cleavage hydrolases; black, regulatory proteins; black hatched, gene encoding a putative ferredoxin oxidoreductase; yellow, genes encoding the two subunits of a ferredoxin-reducing enzyme; white arrows, genes of unknown function, except for *aliA* and *aliB* that are involved in cyclohexane-carboxylate catabolism. Vertical lines mean that the genes are not adjacent in the genome. Clusters IA and II from *G. metallireducens* are indicated. The dashed line in *M. magnetotacticum* indicates that *oah* and *dch* are located at the end(s) of two different contigs and therefore that they might turn out to be linked once the complete genome is assembled. The numbers below the arrows indicate the percentage of amino acid sequence identity between the encoded gene product and the equivalent product from *T. aromatica*.

cluster II, in *Escherichia coli* revealed that it encoded an AMP-forming benzoate-CoA ligase that shares significant amino acid sequence identity with other benzoate-CoA ligases from facultative anaerobes. Considering the strict energy constraints in strictly anaerobic bacteria, it was surprising that an AMP-forming benzoate-CoA ligase is present in iron-reducers, as well as in fermentative (Auburger and Winter, 1992) and sulphate-reducing bacteria (Peters *et al*., 2004), suggesting that the ATP-mediated activation to benzoyl-CoA is a general feature in the anaerobic catabolism of benzoate, regardless of the redox potential of the electron-accepting system.

The key step in anaerobic degradation of benzoyl-CoA is the dearomatization of the benzene ring. The only enzyme known to reduce benzoyl-CoA is the BCR. BCR from *T. aromatica* overcomes energetic limitations by using a low-potential electron donor ferredoxin containing two $[4Fe\text{-}4S]^{+1/+2}$ clusters, and by coupling electron transfer to benzoyl-CoA to a stoichiometric ATP hydrolysis in a manner similar to the well-characterized enzymatic dinitrogen reduction. BCR is an $\alpha\beta\gamma\delta$ heterotetramer (BcrABCD) that contains three cysteine-ligated $[4Fe\text{-}4S]^{+1/+2}$ clusters. Two subunits (α and δ) have two ATP binding sites, and the β and γ subunits form the reductase module that binds benzoyl-CoA (Boll, 2005). A two-subunit ferredoxin-reducing enzyme (KorAB) directly regenerates reduced ferredoxin in *T. aromatica.* The genes encoding the KorAB enzyme in *T. aromatica* and *M. magnetotacticum* are also located within the *bcr* cluster (Fig. 1B) (Dörner and Boll, 2002). In *Azoarcus*, reduced ferredoxin is regenerated by the combined action of a three-subunit NADP $\pm$ dependent 2-oxoglutarate:ferredoxin oxidoreductase (KGOR) and an inducible NADPH:ferredoxin oxidoreductase (putative *bzdV* gene product, Fig. 1B) (Ebenau-Jehle *et al*., 2003; Barragán *et al*., 2004a). BCR from *R. palustris* has been proposed to transfer two additional electrons and form a monoene carbonyl-CoA product, rather than the diene intermediate as BCR from *T. aromatica* (Fig. 1A) (Gibson and Harwood, 2002; Boll, 2005). The *badD* (γ subunit), *badE* (β subunit), *badF* (α subunit) and *badG* (δ subunit) gene products from *R. palustris* show a significant amino acid sequence identity (64–76%) with the corresponding *bcrCBAD* gene products of the BCR from *T. aromatica* and *M. magnetotacticum* (Fig. 1B). On the contrary, the amino acid sequences of the BzdNOPQ proteins, corresponding to the γ, β, δ and α subunits of BCR in *Azoarcus* spp. respectively, differ significantly (22–43% identity) from those of the *bad* and *bcr* gene products (Barragán *et al*., 2004a,b; Boll, 2005). According to this observation, the existence of both *bcr*-type (*T. aromatica, R. palustris, M. magnetotacticum*) and *bzd*-type (*Azoarcus*) BCRs has been proposed (Song and Ward, 2005). Interestingly, Wischgoll *et al*. (2005) did not find homologues of genes encoding the four structural subunits of ATP-dependent BCR in the genome of *G. metallireducens,* and no BCR activity was observed in extracts from cells grown on benzoate when using the established anaerobic radioactive assay and a wide variety of different electron donors. As previously observed in sulphate-reducing bacteria in which specific induction of selenocysteine-containing proteins occurs when the cells use benzoate as the sole carbon source (Peters *et al*., 2004), benzoate degradation by *G. metallireducens* is strictly dependent on the presence of selenium, and molybdenum (or tungstate) appears also to stimulate growth on benzoate (Wischgoll *et al*., 2005). These data strongly suggest that iron- and sulphate-reducing bacteria might have a different enzymology of benzene ring dearomatization than that reported in facultative anaerobes. Based on sequence comparison analyses, Wischgoll and colleagues suggest that the *bamDE* and the *bamCF* gene products form a complex that serves as the electron transfer machinery involved in benzene ring reduction. A selenocysteine residue located near the N-terminus of BamF is predicted, which would explain the observed selenium dependence during growth on aromatic compounds. The *bamG-I* gene products might be involved in complex formation with the *BamC-F* components, transferring the electrons from NAD(P)H for enzymatic dearomatization. The *bamB* gene product shows similarities to tungsten- or molybdenum-containing aldehyde:ferredoxin oxidoreductases. The stimulation of *G. metallireducens* growth by molybdenum in benzoate suggests that functional BamB contains molybdenum (or tungsten), and this protein is suggested to participate in the benzoyl-CoA reducing process (Fig. 1). Thus, Wischgoll and colleagues propose that, unlike the stoichiometric ATP hydrolysis used for benzene ring reduction in facultative anaerobes, a new type of BCR is present in strictly anaerobic bacteria where electron transfer might be driven by a membrane potential.

The *G. metallireducens* BCR-encoding genes become useful for identifying homologous genes in other anaerobes and to explore the biodiversity of the benzoyl-CoA reduction machinery. Recent work using specific primers targeted for polymerase chain reaction (PCR) identification of the *bzd*-type and *bcr*-type BCR-encoding genes in environmental samples revealed that the capacity for anaerobic degradation of aromatic compounds is widespread, but perhaps unrecognized, among cultivated isolates and prokaryotes commonly present in natural environments (Song and Ward, 2005). Denitrifying isolates from the genera *Acidovorax, Bradyrhizobium, Paracoccus, Ensifer* and some *Pseudomonas* strains contained *bcr*-type genes, suggesting that they have been transferred horizontally across different bacterial genera, but only *bzd*-type genes were found in *Azoarcus* isolates. However, BCR-encoding genes were not detected with

any of the *bcr* and *bzd* primers in strains of the *Ochrobactrum* and *Mesorhizobium* genera or in other *Pseudomonas* strains able to metabolize benzoate anaerobically, which suggests that BCR, even within facultative anaerobes, is more diverse than initially thought (Song and Ward, 2005).

In contrast to the observation that a novel type of BCR is present in *G. metallireducens*, the work of Wischgoll *et al*. (2005) reveals that the *bamR, bamQ* and *bamA* gene products show high similarities to the hydratase, dehydrogenase and ring-opening hydrolase respectively, involved in the modified β-oxidation reactions that transform the alicyclic intermediate generated after benzoyl-CoA reduction to 3-hydroxypimelyl-CoA in facultative anaerobes (Fig. 1). This observation suggests that benzoyl-CoA is reductively dearomatized by *G. metallireducens*, most probably in a two-electron reduction, to a dienoyl-CoA compound. A *Thauera*-type central pathway appears to be present therefore in the anaerobic catabolism of benzoate by *G. metallireducens* (Fig. 1A). Another variant of the benzoyl-CoA reduction in which cyclohexene carbonyl-CoA and cyclohexane carbonyl-CoA are produced by a four- or six-electron reduction step has been proposed in fermentative bacteria. Interestingly, the non-aromatic intermediate in these organisms appears to be further metabolized via a *Rhodopseudomonas*-type central pathway (Elshahed *et al*., 2001). To date, genes/proteins involved in benzoyl-CoA reduction and further degradation steps have not been characterized in fermentative bacteria that degrade benzoate.

A global analysis of all gene clusters reported so far for anaerobic benzoate degradation reveals that the genes involved in the modified β-oxidation reactions are adjacent or in close proximity to each other, and that they are associated with BCR-encoding genes in the genome of facultative anaerobes. In contrast, the *bamA* gene (cluster IA) is not linked to the *bamQ* and *bamR* genes (cluster II) in *G. metallireducens* (Fig. 1B). As genes encoding putative transposases flank cluster IA, one could argue that a transposition event separated these genes into two clusters, producing cluster IA linked to genes involved in the lower pathway (cluster IB). Although the gene encoding the AMP-forming benzoate-CoA ligase is usually linked to the rest of the genes of the anaerobic benzoyl-CoA pathway, the corresponding *bclA* gene in *T. aromatica* and *M. magnetotacticum* is linked to genes encoding components of the aerobic pathway for benzoyl-CoA degradation, rather than to the cognate *bcr* anaerobic cluster (Fig. 1B). This observation might reflect the fact that genes encoding benzoate-CoA ligases that act both in aerobic and anaerobic catabolism of benzoate, such as in the case of *T. aromatica* (Schühle *et al.,* 2003), have been recruited to the aerobic cluster instead of to the anaerobic one.

In summary, the results obtained by Wischgoll and colleagues suggest that the major difference in anaerobic benzoate metabolism of facultative and strict anaerobic bacteria is the reductive process for dearomatization of benzoyl-CoA. Further work should focus on the biochemistry of this new reductive process, which could be of great interest in white biotechnology. Moreover, as there are only a few studies on the regulation of clusters involved in anaerobic catabolism of aromatic compounds in facultative anaerobes (Egland and Harwood, 1999; Barragán *et al*., 2005; Rabus *et al*., 2005), the work of Wischgoll and colleagues paves the way to address fundamental questions on how gene expression of the catabolic clusters is controlled in a strict anaerobe.

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