Genetic clues on the evolution of anaerobic catabolism of aromatic compounds

In contrast to the aerobic catabolism of aromatic compounds for which so much information is available, only very little is known about the genes involved in the anaerobic catabolism of aromatics in bacteria. The genes of the central pathway responsible for the anaerobic catabolism of benzoate have been only described in the denitrifying β-Proteobacteria Azoarcus evansii and Thauera aromatica (Breese et al., 1998; Harwood et al., 1999) and in the photosynthetic α-proteobacterium Rhodopseudomonas palustris (Egland et al., 1997; Harwood et al., 1999), and they are organized in catabolic clusters. In all three bacteria, the anaerobic degradation of benzoate begins with its activation to benzoyl-CoA by a benzoate–CoA ligase. However, the subsequent ring reduction to a non-aromatic compound, which is carried out by a four-subunit benzoyl-CoA reductase, and the β-oxidation system that transforms the de-aromatized intermediate by the action of a hydratase, dehydrogenase and ring-cleavage hydrolase, differ between a denitrifying bacterium, T. aromatica, and the photosynthetic bacterium R. palustris. Thus, whereas the T. aromatica pathway converts benzoyl-CoA to 3-hydroxypimelyl-CoA in just four enzymic steps, seven steps are needed through the R. palustris pathway (Harwood et al., 1999; Gibson & Harwood, 2002). The benzoate pathway of A. evansii appears to be similar to that of T. aromatica (Ebenau-Jehle et al., 2003; Harwood et al., 1999). At this point, some questions arise. Is the Thauera-type pathway and the Rhodopseudomonas-type pathway a landmark of anaerobic benzoate catabolism in bacteria with denitrifying and photosynthetic metabolism, respectively? Or, on the contrary, is there a relationship between the type of benzoate degradation pathway and the phylogenetic mark of the organism? To provide some clues as to the answers to the above questions, we decided to expand our knowledge on the genetic determinants responsible for the anaerobic degradation of benzoate in bacteria. We performed an in silico search on the (un)finished microbial genome database at NCBI (http://www.ncbi.nlm.nih.gov/blast/) for genes homologous to those responsible for benzoyl-CoA catabolism in Thauera (Breese et al., 1998), Azoarcus (Schühle et al., 2003) and Rhodopseudomonas (Egland et al., 1997) strains. The only significant match was found with the unfinished genome sequence of Magnetospirillum magnetotacticum MS-1T, which was a surprising finding since this denitrifying strain has not been reported to degrade aromatic compounds (Blakemore et al., 1979). The whole set of genes encoding the putative benzoyl-CoA
Fig. 1. (a) Organization of the gene clusters involved in the anaerobic catabolism of benzoate in *M. magnetotacticum* MS-1<sup>T</sup> (http://genome.jgi-psf.org/draft_microbes/magma/magma.home.html), *A. evansii* (GenBank accession no. AJ428529), *R. palustris* (GenBank accession no. U75363) and *T. aromatica* (GenBank accession no. AJ224959). The predicted functions of the gene products in *A. evansii* and *M. magnetotacticum* are based on the biochemical information reported for the equivalent products in *R. palustris* and *T. aromatica*. Genes are represented by arrows: solid, genes encoding the benzoate–CoA ligases; hatched, genes encoding the four subunits of the benzoyl-CoA reductase and the associated ferredoxins; vertically striped, genes encoding the 2-oxoglutarate:ferredoxin oxidoreductases; cross-hatched, genes encoding enoyl-CoA hydratases; dotted, genes encoding NAD-dependent dehydrogenases; horizontally striped, genes encoding ring-cleavage hydrolases. Open arrows denote genes that do not have a homologue in the *M. magnetotacticum* cluster. Two vertical lines mean that the genes are not adjacent in the genome. Dashed lines between the arrows in *M. magnetotacticum* indicate that the corresponding genes are located at the end(s) of the current contigs (in parentheses). Numbers on top of the arrows indicate the percentage of amino acid sequence identity between the encoded gene product and the equivalent product from *M. magnetotacticum* MS-1<sup>T</sup>. (b) Growth of *M. magnetotacticum* MS-1<sup>T</sup> [obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) as DSM 3856<sup>T</sup>] on benzoate under denitrifying conditions. Cells were incubated for 5 days at 30 °C in minimal medium (Zeyer & Kearney, 1982) containing 3 mM benzoate and 10 mM potassium nitrate. Growth (▲) and benzoate consumption (▼) were monitored spectrophotometrically. (c) Study of the expression of the *bcrA* gene from *M. magnetotacticum* MS-1<sup>T</sup>. RT-PCRs were carried out with the RNAs isolated from benzoate-grown (lane 1) and succinate-grown (lane 3) cells (OD<sub>600</sub> of 0-2) by using primers 3BcrA (5′-CCCCTTCC- CGTCGGCCCTG-3′) and 5BcrA (5′-GATCGACACCCGCTCACCC-3′). The amplified 568 bp fragment from the *bcrA* gene is indicated with an arrow. Lanes 2 and 4 are controls from benzoate- and succinate-grown cells without reverse transcriptase. A PCR from genomic DNA of *M. magnetotacticum* MS-1<sup>T</sup> is shown in lane 5 as a positive control. Lanes M, molecular markers (*HaeIII*-digested *X174* DNA). Numbers on the left represent the sizes of the markers (in bp).
reductase (bcrCBAD), ferredoxin (fdx), hydratase (dch), dehydrogenase (had), ring-cleavage hydrolase (oah), ferredoxin-reducing enzyme (korAB) and benzoate-CoA ligase (bclA) proteins (Breese et al., 1998; Dörner & Boll, 2003; Schühle et al., 2005) were found in the genome of M. magnetotacticum MS-1T (Fig. 1a). It is worth noting that korAB and oah–had are located at the ends of two different contigs in the current genome sequence of M. magnetotacticum and, therefore, it could be possible that such genes are, as in T. aromatica, adjacent to the dch and bcr genes once the complete genome sequence becomes assembled. Interestingly, although the gene encoding the benzoate–CoA ligase is not physically associated with the rest of the genes of the cluster in T. aromatica and M. magnetotacticum, this gene is located within the cluster in R. palustris and A. evansii (Fig. 1a).

The data presented above suggest that M. magnetotacticum MS-1T contains the whole set of genes encoding the pathway for the catabolism of benzoate; therefore, this bacterium should be able to use benzoate as sole carbon and energy source under denitrifying conditions. Fig. 1(b) shows that M. magnetotacticum MS-1T was able to completely deplete 3 mM benzoate after 5 days incubation; the indicator resazurin turned colourless, revealing a strong reducing environment. Moreover, we observed that the ability of M. magnetotacticum MS-1T to degrade aromatic compounds anaerobically was not restricted to the use of benzoate, as it could be extended to the catabolism of 3-hydroxybenzoate, 4-hydroxybenzoate and phenylacetate (data not shown). Therefore, to our knowledge, M. magnetotacticum MS-1T constitutes the first Magnetospirillum strain that has been shown to degrade aromatics under denitrifying conditions, an observation that is in contrast to what has been reported previously (Blakemore et al., 1979).

RT-PCR experiments with M. magnetotacticum MS-1T cells growing under denitrifying conditions using benzoate or succinate as sole carbon sources revealed that bcrA expression was induced when the cells were grown on benzoate but not on succinate (Fig. 1c), which strongly suggests that the bcr gene cluster functions in benzoate metabolism under denitrifying conditions and that expression of such genes is inducible by benzoate. Although anaerobic catabolism of benzoate has been shown in the putative Aquaspirillum sp. strain CC-26 (Shinoda et al., 2000), the set of genes presented in this work constitutes the first one reported for the anaerobic catabolism of benzoate in denitrifying members of the α-Proteobacteria, and it allows us to expand our current knowledge of the anaerobic catabolism of aromatics by denitrifying bacteria, which has been restricted to the β-proteobacterial genera Azorarcus and Thauera before now.

The G+C content of the gene cluster involved in benzoate degradation in M. magnetotacticum MS-1T averaged 63.8 mol%, a value that is very close to the mean G+C content (64 mol%) of the genome, suggesting that this set of genes has been ‘imprisoned’ within the chromosome of this bacterium over a long period of evolution. However, the G+C content (64.5 mol%) of the gene clusters involved in anaerobic benzoate degradation in A. evansii and T. aromatica is slightly lower than that of the host genomes (about 67 mol%) (Anders et al., 1995), which might reflect that the evolutionary origin of such genes in these β-Proteobacteria could be their putative horizontal transfer from an organism with a lower G+C content, such as a Magnetospirillum sp. strain.

Interestingly, comparisons of the global gene arrangement and the deduced amino acid sequences among the anaerobic catabolic clusters reported so far indicate higher similarity between the set of genes from M. magnetotacticum and T. aromatica, two denitrifying micro-organisms, than between genes from M. magnetotacticum and R. palustris (phototrophic bacterium), two members of the α-Proteobacteria (Fig. 1a). Since genetic similarities usually reflect equivalent pathways, our results suggest that it is the type of the electron-accepting system rather than the taxonomic position of the organism that determines the type of anaerobic benzoate degradation pathway, which is in agreement with the observation that the catabolic strategy depends largely on the energy situation of the organism involved and the redox potentials of the electron acceptors that it can use (Peters et al., 2004; Schink et al., 2000). This work also constitutes an example of how in silico analysis of the current genome-sequencing projects is a suitable approach to identify functions/activities that have not been reported previously, for example by revealing M. magnetotacticum as a new model system to study catabolic and regulatory features of the anaerobic metabolism of aromatic compounds.

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