1st Spanish National Conference on Advances in Materials Recycling and Eco – Energy
Madrid, 12-13 November 2009
S02-9

ENGINEERING BIOCATALYSTS FOR THE ANAEROBIC RECYCLING OF TOXIC AROMATIC HYDROCARBONS

M.T. Zamarro, M. Carmona, A. Valencia, M.J.L. Barragán, B. Blázquez, J.L. García and E. Díaz

Centro de Investigaciones Biológicas (CSIC). Ramiro de Maeztu 9. 28040 Madrid, Spain

Abstract

Azoarcus sp. CIB is a denitrifying betaproteobacterium that uses different aromatic compounds, including toxic hydrocarbons such as toluene and *m*-xylene, as sole carbon sources, and is susceptible of genetic manipulation. To acquire a global view of all the genetic determinants involved in the anaerobic catabolism of aromatic compounds in strain CIB, and to accelerate and complete our understanding about this anaerobic catabolism, we have performed a genomic survey in the recently sequenced genome of this strain. This information paves the way for a pathway engineering approach within the field of anaerobic degradation of aromatics.

Keywords: anaerobic catabolism, aromatic compounds, metabolic engineering, gene cassette

INTRODUCTION

The removal of pollutants from the environment via natural physico-chemical and biological processes (natural attenuation) is, in general, a slow and unpredictable way of counteracting anthropogenic pollution and irreversible damage to the biosphere. Therefore, the main, if not the only, successful strategy to fight pollution is the use and manipulation of the detoxification abilities of living organisms (bioremediation) [1]. Although most organisms are endowed with detoxification abilities (*e. g.*, mineralization, transformation or immobilization of pollutants) microorganisms, particularly bacteria have been the most studied and the most frequently used materials for bioremediation strategies [2].

Next to glucosyl residues, the benzene ring is the unit of chemical structures most widely spread in nature. Moreover, the thermodynamic stability of the benzene ring has contributed to the widespread production and industrial usage of natural and xenobiotic aromatic compounds, but persistence of these compounds, many of which are toxic, after their release into the environment, poses a major environmental problem [3].

Microorganisms play a crucial role in biogeochemical cycles and in a sustainable development of the biosphere. Bacteria have evolved to degrade most naturally occurring organic compounds, including the persistent aromatics.

The bacterial catabolism of aromatic compounds involves a wide variety of peripheral pathways that activates structurally diverse substrates into a limited number of common intermediates that are further cleaved and processed by a few central pathways to the central metabolism of the cell [4].

There are two major strategies to degrade aromatic compounds depending of the presence or absence of oxygen. In the aerobic catabolism of aromatics, the oxygen is not only the final electron but also a co-substrate for the hydroxylation and oxygenolytic ring cleavage of the aromatic ring. In contrast, the anaerobic catabolism of aromatic compounds uses a completely different strategy, based on reductive reactions, to attack the aromatic ring. While the aerobic catabolism of aromatic compounds has been studied for several decades, the anaerobic degradation of aromatic compounds is a more recently discovered microbial capacity that still awaits a deeper understanding. However, anoxic conditions dominates in many natural habitats and contaminated sites (*e. g.*, aquifers, aquatic sediments and submerged soils) where the biodegradation is carried out by anaerobes using alternative final electron acceptors such as nitrate, sulfate or ferric ions[5].

EXPERIMENTAL

Bacterial growth conditions

Azoarcus sp. CIB was grown anaerobically at 30ºC on MC medium and 10 mM potassium nitrate as described previously [6]. As carbon source benzoate was added to 3 or 10 mM and toluene was added to 500mM in a heptametylnonane phase. The concentration of benzoate in the culture medium was spectrophotometrically monitored at 273 nm.

Molecular biology techniques

Recombinant DNA techniques were carried out by published methods [7]. Plasmid DNA was prepared with a High Pure plasmid isolation kit (Roche).
DNA fragments were purified with Gene-Clean Turbo (Q-BIOgene). Oligonucleotides were supplied by Sigma. Plasmids were transferred from *Escherichia coli* S17-1λpir (donor strain) into *Azoarcus* sp. recipient strain by biparental mating as described previously [6].

Gas chromatography-mass spectrometry analysis

Polyhydroxybutyrate (PHB) production was quantified by GC-MS of the metanolyzed polyester as previously described [8]

Phase contrast and electron microscopy

Phase contrast image acquisition of PHB granules within the cell has been taken using an optical Nikon OPTIPHOT microscope and the UFX-IIA photomicrographic attachment with a 100X objective magnification. Electron image acquisition of PHB granules within the cell has been taken using a transmission electron microscope JEOL JEM 1230.

Previously, cells were fixed in 5% (w/v) glutaraldehyde, included with 2.5% (w/v) OsO4 and embedded in Epon 812 resin. Ultrathin sections were cut with a microtome using a Diatome diamond knife.

RESULTS AND DISCUSSION

Despite the great environmental relevance of the anaerobic catabolism of contaminants, many biochemical and genetic aspects as well as their biotechnological applications are still unexplored. Different strains of the genus *Azoarcus* are able to degrade under anaerobic conditions toxic compounds such as aromatic hydrocarbons. *Azoarcus* sp. CIB is one of such strains that uses toluene and *m*-xylene as sole carbon sources and is susceptible of genetic manipulation.

To have a global view of all the genetic determinants involved in the anaerobic catabolism of aromatic compounds in strain CIB, we have performed a genomic survey in the recently sequenced genome of this strain. For the first time, we have identified in the same organism three different gene clusters, *i. e., bzd*, *hbd* and *mbd*, encoding the corresponding benzoyl-CoA, 3-hydroxybenzoyl-CoA and 3-methylbenzoyl-CoA central pathways, respectively. A wide variety of gene clusters encoding peripheral pathways responsible for the anaerobic catabolism of different aromatic acids, amino acids, alcohols, phenols and hydrocarbons to the cognate central pathways, have been also identified.

A number of regulatory genes likely to be involved in the specific regulation of each particular gene cluster have been also determined. Taken together, all these analyses reveal *Azoarcus* sp. CIB as a good model system to study the genetics and regulation of the anaerobic metabolism of aromatic compounds.

On the other hand, the anaerobic gene clusters from *Azoarcus* sp. CIB constitute also a source of new genetic tools with a number of applications in the field of environmental biotechnology. In this sense, we have engineered the first broad-host range gene cassette (pLB1) containing the *bzd* gene cluster for the anaerobic degradation of benzoate [6]. The *bzd* cassette has been used to expand the ability of some denitrifying bacteria to use benzoate as sole carbon source under anaerobic conditions. Moreover, the multicopy gene dosage *bzd* cassette is being used to increase the efficiency of the anaerobic degradation of aromatic compounds in *Azoarcus* sp. CIB. Since *Azoarcus* sp. CIB is able to produce PHB when cultured with toxic aromatic compounds such as toluene, an enhanced expression of the *bzd* genes in pLB1 may lead to an increased production of this bioplastic [9]. Pathway engineering is a novel strategy in the field of anaerobic biodegradation of aromatics and validates the use of *Azoarcus* sp. CIB

and its catabolic genes for the development of new recombinant biocatalysts in white biotechnology.

Although microorganisms have acquired the ability to use pollutants as carbon and energy sources, their efficiency at removing such pollutants might not be optimal for cleaning up present-day pollution. In fact, microorganisms have evolved toward ecological fitness rather than biotechnological efficiency [10]. To enhance the metabolic efficiency of a microorganism for a particular environmental application, engineering should be carried out.

The genetic and recent genomic and proteomic approaches that have been undertaken to study anaerobic catabolism of aromatic compounds have contributed significantly to accelerating and completing our understanding of different aspects of the physiology, ecology, biochemistry and regulatory mechanisms of the catabolic pathways. Hence, these advances will became crucial to recreate and accelerate natural processes in the test tube as well as to accomplish their rational manipulation to design more efficient biocatalysts for different biotechnological applications that include bioremediation of polluted sites or biotransformation of toxic compounds into fine chemicals or other high added-value products [5].

REFERENCES

[1] Lovley DR (2003) Cleaning up with genomics: applying molecular biology to bioremediation. *Nat Rev Microbiol* 1: 35-44

[2] Dua M, Singh A, Sethunathan N and Johri AK (2002) Biotechnology and bioremediation: successes and limitations. *Appl Microbiol Biot* 59: 143-152

[3] Dagley S (1986) Biochemistry of aromatics hydrocarbons degradation in Pseudomonads. In Sokatch J and Ornston LN (eds) The Bacteria. Academic Press, Orlando, Vol 10, pp 527-555

[4] Díaz E (2004) Bacterial degradation of aromatic pollutants: a paradigm of metabolic versatility. *Int Microbiol* 7: 173-180

[5] Carmona M, Zamarro MT, Blázquez B, Durante-Rodríguez G, Juárez JF, Valderrama JA, Barragán MJL, García JL and Díaz E (2009) Anaerobic catabolism of aromatic compound: a genetic and genomic view. *Microbiol Mol Biol Rev* 73: 71-133

[6] Barragán MJL, Carmona M, Zamarro MT, Thiele B, Boll M, Fuchs G, García JL and Díaz E (2004) The *bzd* gene cluster, coding for anaerobic benzoate catabolism, in *Azoarcus* sp. strain CIB. *J Bacteriol* 186: 5762-5774

[7] Sambrook JW and Russell DW (2001) Molecular Cloning: a Laboratory Manual 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory

[8] Lageveen RG, Huisman GW, Preusting H, Ketelaar P, Eggink G and Witholt B (1988) Formation of polyesters by *Pseudomonas oleovorans*: Effect of substrates on formation and composition of poly-(R)-3-hydroxyalkanoates and poly-(R)-3-hydroxyalkenoates. *Appl Environ Microbiol* 54: 2924-2932

[9] Prieto MA, de Eugenio LI, Galán B, Luengo JM and Witholt B (2007) Synthesis and degradation of polyhydroxyalkanoates. In *Pseudomonas*: a Model System in Biology. *Pseudomonas*, vol. V, Eds, Ramos JL and Filloux A. Springer. 397-428

[10] Schmid A, Dordick JS, Hauer B, Kiener A, Wubbolts M and Witholt B (2001) Industrial biocatalysis today and tomorrow. *Nature* 409: 258-268