Basic knowledge and perspectives of bioelimination of xenobiotic compounds

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Abstract

Almost every natural product, irrespective of its molecular weight or structural complexity, is degraded by one or another microbial species in some particular environment. The omnipotence of microorganisms also extends to the majority of synthetic compounds, which are funneled into the natural metabolic cycles. Certain substituents such as halogen, sulfo-, azo- or nitro-groups, particularly the accumulation of such groups and specific substitution patterns confer xenobiotic character to a synthetic compound. Moreover, the electron-withdrawing character of these substituents generates electron deficiency and thus makes the compounds less susceptible to oxidative catabolism. As a consequence, many of these chemicals tend to persist under aerobic environmental conditions.

When enzymes with low substrate specificity encounter foreign compounds, highly reactive species may be generated. On the one hand, these can eliminate some of the xenophors spontaneously generating less persistent metabolites. On the other hand, gratuitous metabolism of complex structures may give rise to extensive chemical misrouting, generating dead products of high molecular weight. Biodegradation of a xenobiotic substance can be accomplished when the catabolic activities, present in mixed microbial communities, complement each other. Thus, syntrophic interactions can lead to complete mineralization of even complex xenobiotic compounds.

Mineralization of xenobiotics by a single organism can be achieved by taking advantage of natural or induced gene transfer to construct hybrid degradative pathways. Mobilization of blocks of genes, encoding catabolic bottleneck reactions, may speed up the evolutionary potential of natural communities so that under appropriate selective conditions new multifunctional pathways are generated.

As a consequence of the electron deficiency of xenobiotic compounds such as polychlorinated arenes or ethenes, azo dyes or polynitroaromatics, the combination of the reductive potential of anaerobic microbes with subsequent oxidative processes opens a hitherto largely unexploited technology for biological treatment of waste water and for soil bioremediation. Copyright © 1996 Elsevier Science B.V.

Keywords: Anaerobic/aerobic processes; Arylsulfonates; Azodyes; Cometabolism; Electron deficient xenobiotics; Haloalkenes; Humification

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1. Introduction

Natural communities of microorganisms harbor an amazing physiological versatility and catabolic potential for the breakdown of an enormous number of organic molecules. Almost every natural product, irrespective of its molecular weight or structural complexity, is readily degraded by one or another microbial species in some particular environment. This omnipotence of microorganisms also extends to the majority of synthetic compounds which are funneled into the natural metabolic cycles. Certain substituents such as halogen, sulfo-, azo- or nitro-groups (Fig. 1), particularly the accumulation of such groups and specific substitution patterns, confer xenobiotic character to a synthetic compound. Moreover, the electron-withdrawing character of these substituents generate an electron deficiency and thus make the compounds less susceptible to oxidative catabolism.

In general, oxygen is directly involved in the initial attack of aliphatic and aromatic compounds. Because introduction of hydroxyl functions into the molecule is necessary for further oxidative catabolism, the initial electrophilic attack by oxygenases of aerobic bacteria may become a rate-limiting step. This can be demonstrated in bacterial systems, exhibiting low substrate specificity of oxygenases so that a strict correlation between the Hammett substituent constant and the log $V_{rel}$ values of the initial dioxygenation reaction can be observed (Reineke and Knackmuss, 1978).

The fate of environmental pollutants is largely determined by abiotic processes such as photooxidation and by the metabolic activities of microorganisms. Since catabolic enzymes are more or less specific, they can act on more than their natural substrate. This explains why the majority of xenobiotics are subject to fortuitous metabolism (cometabolism). Normally, an initial catabolic enzyme or sequence of enzymes convert a chemical to an organic product that is not further metabolized. Thus, for a cometabolically active organism, the process is unproductive because it is not coupled to energy conservation. Therefore, in a natural mixed population, a wide variety of dead-end products (Fig. 2) may be accumulated and subject to physical and chemical secondary reactions. Since initial oxidation by mono- or dioxygenases requires reduction equivalents or the loss of active protein by suicide inactivation, cometabolism may
Unproductive

![Chemical Reaction](image)

or

Counterproductive

![Chemical Reaction](image)

1,2- or 1,4-additions, condensations, radical reactions

Fig. 3. Cooxidation of 2-chlorophenol by phenol-degrading bacteria generates 3-chlorocatechol, which cannot be utilized (unproductive catabolism is indicated by heavy arrows). If 3-chlorocatechol is subject to meta-cleavage, a highly reactive acyl chloride may be generated which destroys the meta-cleavage enzyme by suicide inactivation. As a consequence, 3-chlorocatechol is accumulated and, by autoxidation, quinones or phenoxyl radicals may be formed. Chemical misrouting and the toxicity of the autoxidation products make the catabolism of 2-chlorophenol even counterproductive (indicated by broken arrows).

be even counterproductive. An example is the cooxidation of 2-chlorophenol by phenol- or cresol-degrading microorganisms, which gives rise to the accumulation of 3-chlorocatechol (Fig. 3) (Schmidt et al., 1983). The latter, if subject to meta-cleavage, generates an arylchloride which irreversibly inactivates the ring cleavage enzyme (Bartels et al., 1984). As a consequence, chlorocatechols are accumulated which by autoxidation generate highly reactive products such as quinones or phenoxyl radicals. These are subject to chemical misrouting or may be toxic and thus paralyze the entire process.

Cometabolic transformation of a xenobiotic compound often generates reactive metabolites such as epoxides, dihydrodiols, aromatic diols, aromatic amines (see below) or at least products that are more easily oxidized than the original chemical. On the one hand, the reactive metabolite may be misrouted by spontaneous chemical or physical reactions giving rise to absorption, coupling or polymerization reactions (Fig. 4). On the other hand, further chemical or biological transformation may eliminate the xenophor so that at least part of the xenobiotic compound can be mineralized and utilized by certain members of the population.

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Fig. 4. In general cometabolic transformation of a xenobiotic compound represents an activation of the molecule. Subsequent spontaneous reactions can therefore lead to chemical misrouting (see Fig. 3) or anionic elimination of the xenophor allows productive metabolism of the chemical.
2. Electron-deficient xenobiotics

Because of the electron-withdrawing character of the xenophors given in Fig. 1, anaerobic microorganisms harboring strongly reducing nucleophiles should react with these structures, generating less electrophilic metabolites. Candidates for these initial reductive transformations are polyhalogenated compounds which may be dehalogenated. These reactions would involve the anionic removal of a halogen and its replacement by a hydride ion (Fig. 5). Actually, reductive dehalogenation occurs most readily under strict anaerobic conditions ($\leq -400 \text{ mV}$) and requires a reducing auxiliary substrate. Recently, it has been shown that this exergonic dehalogenation may be coupled to energy conservation (Mohn and Tiedje, 1992). With a pure culture of *Dehalospirillum multivorans*, growth in mineral medium could be demonstrated, with $H_2$ plus perchloroethane (PCE) as sole source of energy when acetate served as a carbon source (Neumann et al., 1994). This clearly indicates a chemiosmotic mechanism of energy conservation. Since dechlorination yields cis-dichloroethene and vinylchloride, the highly electron-deficient tetrachlorethene, which cannot be attacked by aerobic bacteria, is converted to compounds that are less electron-deficient and readily degradable by cooxidation (Ewers et al., 1990). It is obvious that the combination of a reductive and an oxidative process offers a hitherto unexploited possibility for complete degradation of polychlorinated hydrocarbons as contaminants of soil and groundwater.

A similar situation exists in polynitroaromatic compounds and in azo dyes (Fig. 5). The electron-accepting character of the azo- and nitro-group renders these chemicals less susceptible to oxidative processes. In contrast, with increasing number of nitro-groups on the aromatic ring, initial reductive transformations are favored.

Because of the strong electron deficiency of polynitroaromatics, even aerobic bacteria exhibit reductive potentials against these xenobiotics. An unusual hydrogenation of the aromatic nucleus has been recently discovered for the degradation of picric acid and 2,4,6-trinitrotoluene (TNT) (Rieger and Knackmuss, 1995). During coreduction of the nitro-groups of TNT, amino-groups are generated. These weaken the electron deficiency originally present in the aromatic ring so that the reaction velocities of the sequential reduction of TNT decrease with the number of nitro-groups being converted into amino-groups. Therefore, complete reduction of TNT to 2,4,6-triaminotoluene (TAT) requires strict anaerobic conditions ($\leq -200 \text{ mV}$) and a reductant as an auxiliary substrate such as glucose or saccharose.

TNT is thus completely reduced to TAT by anaerobic sludge with aminodinitro- and diaminonitrotoluenes as intermediates (Rieger and Knackmuss, 1995). TAT is an electron-rich compound and should be readily oxidized when treated aerobically. If TNT-contaminated soil is treated anaerobically, TNT is also subject to stepwise reduction; however, the end product TAT is not detectable. Kinetic data clearly demonstrate that irreversible sorption of TAT proceeds already under strict anaerobic conditions. Obviously, the acidic polyanion structures of the clay minerals and of the humic substances interact with the basic groups of TAT. In addition, subsequent $O_2$-dependent reactions and aerobic conditions may give rise to the formation of polymers, which resembles humification of natural phenolic sub-
Fig. 6. Biological remediation of TNT-contaminated soil by anaerobic/aerobic treatment. Glucose or saccharose was added as an auxiliary substrate which is degraded by the autochthonous soil population. Part of the reducing equivalents were used for stepwise reduction of TNT via aminodinitro- and nitrodiamino toluenes (ADNT and DANT). Triaminotoluene interacts irreversibly with the clay and humic acid fraction of soil.

Fig. 6 summarizes the process of anaerobic/aerobic treatment of TNT-contaminated soil. It offers a cost-effective and simple way to clean up polluted sites and does not require the establishment of special nonindigenous microorganisms (Daun et al., 1995). Ecotoxicological tests with aquatic and terrestrial organisms indicate that toxic effects cannot be detected in the remediated soil and it was thus recommended for reuse as green land and recreation areas (Lenke et al., unpublished).

3. Degradation of xenobiotics by mixed cultures

With increasing complexity of a xenobiotic, we cannot expect to find complete catabolic pathways in a single organism. Besides incomplete oxidation and accumulation of dead-end metabolites, a higher degree of biodegradation and even mineralization can be expected when cometabolic activities within a microbial community complement each other (Fig. 2, sequences a–c + d + e–g). Such syntrophic interactions actually exist in natural populations and in certain cases the concerted action of a two-species culture is well understood (Fig. 7). A case in point is the degradation of naphthalenesulfonates (Fig. 8), which

Fig. 7. Degradation of a xenobiotic compound by a synergistic two-species culture. Organism I eliminates the xenophor as an anion and utilizes part of the carbon skeleton, whereas organism II harbors a complementary sequence that allows complete mineralization and productive catabolism of the dead-end product of organism I.
Fig. 8. Degradation of naphthalene-2-sulfonate (2NS) in syntrophic culture. Strain BN6, tentatively identified as a Sphingomonas strain, can partially degrade 2NS to salicylate as a toxic dead-end metabolite. Strain BN6 can only grow with 2NS if a salicylate-degrading strain is present.

are structural features and building blocks of azo dyes.

Sphingomonas strain BN6 harbors the ability to degrade naphthalene-2-sulfonate (2NS) to salicylate. Because the latter compound is not further degraded and toxic to strain BN6, it can grow with 2NS only in the presence of a salicylate-assimilating organism. The initial catabolic enzymes of 2NS catabolism distinguish themselves by an extraordinarily low substrate specificity so that a broad spectrum of substituted amino- and hydroxynaphthalene sulfonates are transformed into the corresponding amino- and hydroxysalicylates (Fig. 9). Therefore, the functionality of the system can easily be expanded by complementary organisms degrading substituted salicylates. Typically, it is difficult to establish a stable continuous degradation process with these mixed cultures be-

Fig. 9. Turnover of amino- and hydroxynaphthalene-2-sulfonates by Sphingomonas strain sp. BN6. With the exception of 3-substituted isomers and 5-amino-naphthalene-2-sulfonate, these xenobiotics were oxidized to the corresponding salicylates (Nortemann et al., 1986). 5-Aminonaphthalene-2-sulfonate is also readily cooxidized to 5-hydroxyquinoline-2-carboxylate (Nortemann et al., 1993) as a dead-end product.

cause interspecies transfer of the salicylates, particularly of the amino- and hydroxysalicylates, may be disturbed by autoxidation and chemical misrouting of these metabolites. The instability of the biological system can be circumvented by immobilization of the complementary microorganisms (Diekmann et al., 1988) or by generating
Table 1
Competition between the mixed culture and the hybrid strain BN6 (pWW60-3026) during growth with 2NS

<table>
<thead>
<tr>
<th>Days</th>
<th>Strains (cells ml$^{-1}$)</th>
<th>Rel. cell number of hybrid strain (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BN6 total</td>
<td>BN6 wild type</td>
</tr>
<tr>
<td>0</td>
<td>2.3 x 10$^9$</td>
<td>1.3 x 10$^9$</td>
</tr>
<tr>
<td>0.94</td>
<td>4 x 10$^9$</td>
<td>3 x 10$^9$</td>
</tr>
<tr>
<td>1.93</td>
<td>2.6 x 10$^9$</td>
<td>1 x 10$^9$</td>
</tr>
<tr>
<td>2.83</td>
<td>2.3 x 10$^9$</td>
<td>2.3 x 10$^7$</td>
</tr>
<tr>
<td>3.84</td>
<td>4.3 x 10$^9$</td>
<td>&lt;4.3 x 10$^7$</td>
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<td>&lt;4.1 x 10$^7$</td>
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<td>&lt;3.6 x 10$^7$</td>
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</tr>
<tr>
<td>8.1</td>
<td>3.8 x 10$^9$</td>
<td>&lt;3.8 x 10$^6$</td>
</tr>
<tr>
<td>9.6</td>
<td>4 x 10$^9$</td>
<td>&lt;4 x 10$^6$</td>
</tr>
<tr>
<td>11</td>
<td>4.4 x 10$^9$</td>
<td>&lt;4.4 x 10$^6$</td>
</tr>
<tr>
<td>12.7</td>
<td>2.6 x 10$^9$</td>
<td>&lt;2.6 x 10$^6$</td>
</tr>
</tbody>
</table>

hybrid strains, which harbor a complete catabolic sequence (see Fig. 2, sequence a–c + h–k).

4. Evolution of hybrid pathways

The experiences with haloaromatic compounds have shown that new hybrid degradative capabilities for a broad spectrum of mono- and dichlorinated compounds such as chlorobenzenes (Y = H), chlorobenzoates (Y = COOH), chlorophenols (Y = OH), chloroanilines (Y = NH$_2$) or chlorobiphenyls (Y = phenyl) can be evolved by natural gene transfer (Fig. 10) (for review see Gottschalk and Knackmuss, 1993). The basis for this evolutionary potential is, on the one hand, the catabolic potential of bacteria that utilize aromatic and methyiaromatic compounds and cooxidize the corresponding chloroaromatic structure to chlorocatechols. On the other hand, bacteria harboring the complementary catabolic sequence for the assimilation of chlorocatechols are rare but ubiquitous in natural populations and can be enriched by chlorinated aromatics that exhibit low toxicity. Thus, by use of 3-chlorobenzoate or 2,4-dichlorophenoxycetate, strains were enriched that harbor the chlorocatechol-assimilating potential on transferable genetic elements such as the plasmids pJP4, pWR1 or pAC25. Horizontal transfer of these plasmids explains why hybrid pathways for chlorosubstituted aromatic compounds can be evolved in the laboratory and during adaptation of the microflora of activated sludge.

In the case of sulfonated naphthalenes, hybrid strains with a complete catabolic sequence should be generated by conjugative transfer to strain BN6 of naturally existing plasmids that encode salicylate degradation. This approach has not been successful because the well known plasmids NAH7, SAL or pWW60 were not transferred to Sphingomonas sp. BN6. In contrast, broad host range plasmids from the incompatibility group Inc Pl or Inc P4 were readily transferred from Escherichia coli to strain BN6 (10$^{-3}$–10$^{-4}$ per donor cell). Therefore, the constructed plasmid pWW60-3026 harboring the genes of salicylate degradation plus a regulatory element from NAH (Pseudomonas putida NCIB9816) were successfully transferred to strain BN6 (Ruß et al., unpublished). Derivative strains, when transferred to mineral medium with 2NS as sole source of carbon and energy, could grow with 2NS but exhibited only slightly higher catabolic performance when compared with the mixed culture consisting of Sphingomonas sp. BN6 and Pseudomonas putida NCIB9816. Neither growth characteristics with 2NS in continuous culture nor comparison
of key catabolic enzymes in crude extracts of cells of the two species and the hybrid culture could clearly demonstrate the advantage of the hybrid strain. A competition experiment with 2NS as the limiting substrate was therefore carried out in continuous culture. The complementary system of strain BN6 and strain NCIB9816 competed with BN6 (pWW60-3026) harboring the hybrid pathway of 2NS catabolism. Surprisingly, within 4–5 days, the hybrid organism clearly dominated and completely replaced the two-species culture (Table 1).

5. Perspectives

These preliminary observations with 2NS-degrading hybrid bacteria clearly showed that genetic optimization and construction of hybrid strains may increase the rate and extent of removal of xenobiotic compounds in a continuous process.

The functionality of Sphingomonas sp. BN6 towards a broad spectrum of substituted naphthalenes (Fig. 11) can even be expanded to sulfonated azo dyes. This, however, requires a two-step anaerobic/aerobic process in which the bacterial 2NS-grown biomass of the Sphingomonas sp. BN6 and the salicylates-degrading strains is used for gratuitous reduction of sulfonated azo dyes (Haug et al., 1991). Thus, under anaerobic conditions and in the presence of an auxiliary electron donor, Mordant yellow is subject to complete cleavage to the corresponding aromatic amines, which are completely mineralized by the subsequent aerobic step. Characteristically, only cells of strain BN6 grown in the presence of naphthalene sulfonates can reduce azo dyes. This indicates that an uptake system is induced that gratuitously takes up sulfonated azo dyes, which are coreduced by an azo reductase. This latter activity is constitutive and rate-limiting in BN6, so that for practical purposes it may be necessary to improve its expression or recruit a more efficient inducible azo reductase.

The modular nature of the catabolic sequence of sulfonated aromatic compounds and azo dyes and its acquisition together with an appropriate regulatory unit by salicylates-degrading organisms would certainly lead to a burst of evolutionary potential for new and highly efficient hybrid routes of these xenobiotics (Fig. 11). The mobilization of the genes of the initial catabolic sequences, at least for substrate uptake and dioxygenolytic desulfonation, would create an evolutionary potential as described above for the haloaromatics (Fig. 10). Previous work has shown that aromatics- and methylaromatics-degrading organisms in natural populations readily acquire the genetic module of chlorocatechol assimilation and thus evolve novel biochemical routes, not only for single compounds but more importantly for mixtures of haloaromatic xenobiotics.

In the case of sulfonated naphthalenes, the genetic determinants of the initial catabolic sequence have to be mobilized because these are unique and multifunctional. Acquisition of this block of genes by ubiquitous salicylates-degrading
Fig. 12. Biodegradation of azo dyes could be accomplished by bacteria harboring a highly efficient uptake and azo reductase system that are used in a two-step anaerobic/aerobic process. The hybrid arylsulfonates degraders (see legend of Fig. 11) must suppress chemical misrouting of the sulfonated o- and p-aminohydroquinones that are generated by the initial cleavage of the azo bond.

This exciting area of experimental evolution holds much promise to overcome biological limitations in bioremediation processes. In particular, future work has to focus on the constraints that are due to misrouting of metabolites by incomplete pathways, substrate bioincompatibilities and insufficient performance of certain naturally existing catabolic potentials.