Microbial degradation of chlorinated compounds. Application of specialized bacteria in the treatment of contaminated soil and waste water.

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SUMMARY

Man-made chemicals used as refrigerants, fire retardants, paints, solvents, and pesticides cause considerable environmental pollution and human health problems as a result of their persistence, toxicity, and transformation into hazardous metabolites. Many environmentally important xenobiotics, introduced for industrial use, are chlorinated, and chlorination often is implicated as a reason for persistence.

The biological cleanup of waste streams, groundwater, or soil contaminated with these compounds is an attractive alternative for other treatment technologies. For this, it is essential that efficient biodegradation of chlorinated organic compounds to harmless products can be achieved.

The development of (aerobic) treatment technologies for polluted environments and waste streams will require an understanding of the microbial potential and the ecophysiology of the most suitable organisms. Therefore, we have studied physiological pathways and some kinetic aspects of the biotransformation of chlorinated aliphatics and some aromatics. Special emphasis was given to the identity and kinetics of dechlorination reactions since this is the step where toxicity is lost.

Organisms using xenobiotic compounds for growth: stimulation of degradation in soil

Chapter 2 describes the isolation and testing of several bacterial strains that utilize industrial solvents for their ability to degrade chlorinated and non-chlorinated hydrocarbons in soil. We have found that addition of a mixture of benzene-, toluene-, xylenes-, chlorobenzene-, and 1,2-dichlorobenzene-degrading bacteria significantly stimulates conversion of these chemicals in soil slurries. The results showed that degradation was due to growth of the inoculated cells using the aromatic compounds as carbon and energy source. Addition of non-specialized inoculum (activated sludge) did not stimulate degradation. Furthermore, the effect of inoculation of soil slurries with two pure bacterial cultures that utilize dichloromethane (*Hyphomicrobium* GJ21 and *Methylobacterium* DM4) was tested and stimulated degradation of dichloromethane was observed.

Cometabolism: the role of methanotrophs

No organisms have yet been isolated that utilize chlorinated aliphatic hydrocarbons containing more than two chlorines per molecule. Cometabolic conversions under aerobic conditions are possible, however, and a number of different organisms have been investigated for this purpose. We have chosen to use methanotrophic bacteria since these organisms show much higher conversion rates than other mono- or dioxygenase producing microorganisms.

Chapter 3 describes our first degradation experiments with mixed and pure cultures of methanotrophic bacteria. Out of seven chlorinated aliphatic hydrocarbons tested, only *trans*-1,2-dichloroethylene (t12DCE) was relatively non-toxic for a growing mixed culture of methanotrophs and was degraded at a rate of 3 nmol min^{-1} mg of $cells^{-1}$. From the consortium, a pure culture of a strain of *Methylomonas* (strain GJ6) was isolated and found to be capable of degradation of t12DCE when grown in the presence of methane or

methanol. Continuous degradation of t12DCE (1.2 nmol t12DCE min^{-1} mg of $cells^{-1}$) was observed in a fermentor in which the *Methylomonas* strain was grown in the presence of methane.

Methanotrophic bacteria are able to produce at least two types of methane monooxygenase (MMO). All methanotrophs studied so far are able to produce a membrane-bound or particulate methane monooxygenase (pMMO). The enzyme contains copper, and therefore methanotrophs that only produce this enzyme require copper for growth. Some methanotrophs, however, are also able to produce a soluble type methane monooxygenase (sMMO) that is derepressed, if copper is absent from the growth medium.

In Chapter 4 it was shown that many chlorinated compounds such as trichloroethylene (TCE), one of the most important chlorinated hydrocarbons, was rapidly degraded by high density suspensions of *Methylosinus trichosporium* OB3b cultivated under copper stress and thus expressing sMMO.

Chapter 6 describes similar degradation experiments, using formate as an electron donor and high density suspensions of *Methylomonas* strain GJ6. TCE was poorly degraded, showing that pMMO of this strain is not active with TCE. The lack of activity appeared not to be caused by product inactivation, as with *Methylosinus trichosporium* OB3b, but was the result of the limited substrate range.

The results described in Chapter 4 and 6 are summarized in Table 6.3. This table lists some compounds that we found to be substrates for either only sMMO (from *Methylosinus trichosporium* OB3b) or both sMMO and pMMO (from *M. trichosporium* OB3b or *Methylomonas* strain GJ6, which gave essentially identical results).

Kinetics and toxicity of cometabolic conversions

Chapter 5 describes the kinetics of the degradation of TCE and seven other compounds by *Methylosinus trichosporium* OB3b expressing sMMO. Compounds that were readily degraded included chloroform, t12DCE, and TCE, with V_{max} values of 550, 330, and 290 nmol min^{-1} mg of $cell^{-1}$, respectively. TCE was found to be toxic for the cells, and this phenomenon was studied in detail. Addition of activated carbon decreased the acute toxicity of high levels of TCE by adsorption, and slow desorption enabled the cells to partially degrade TCE. TCE was also toxic by inactivating the cells during conversion. The degree of inactivation, which is described by the inactivation constant, was proportional to the amount of TCE degraded; maximally 2 μmoles of TCE could be converted per mg of *M. trichosporium* OB3b cells. During conversion of [^{14}C]TCE, various proteins became radiolabeled, indicating nonspecific covalent binding of degradation products to cellular proteins rather than a specific suicidal reaction catalyzed by the enzyme.

TCE degradation in continuous culture

In Chapter 7, TCE degradation was further investigated in continuous cultures of *M. trichosporium* OB3b. The organism was grown in a copper-limited medium to which methane as a growth substrate and TCE were continuously added via the gas phase. It was found that at an incoming TCE concentration of 0.55 μmol (73 μg) liter^{-1} of air, 97% of the TCE was degraded. This resulted in an elimination capacity of 25 nmol TCE min^{-1} liter^{-1} (0.2 g TCE h^{-1} m^{-3}) of reactor volume. The degradation rate of TCE was 0.024 nmol min^{-1} mg of cells^{-1} (24 mU g of cells^{-1}). Increase of the incoming TCE concentration to 3.1 μmol (0.4 mg) liter^{-1} of air led to washout of the culture.

CONCLUDING REMARKS

Specialized cultures as described in this thesis will become increasingly important for the application of biological techniques for environmental protection and cleanup. With compounds that support growth of microorganisms that perform catabolic reactions, the approach seems straightforward. Our results show that (under optimal conditions), inoculation with low amounts of these specific microbial strains was sufficient to initiate rapid degradation in non-sterile soil and growth was not inhibited by the indigenous microflora.

Cometabolic oxidation seems to be the only option for aerobic transformation of compounds such as chloroform, dichloroethylenes, and TCE. Therefore, an important goal will be to achieve biological removal of these xenobiotics by microorganisms that rely on cometabolic conversion. The applicability of methanotrophs for the removal of TCE and related compounds will be complicated, since for rapid degradation it is needed to specifically stimulate growth of methane utilizing bacteria producing sMMO. Copper limitation is known to play an essential role, and this can possibly be used if removal from the gas phase with a bioreactor has to be achieved. The availability of copper will be much more difficult to manipulate in a soil or groundwater environment. Another problem could be removal of high concentrations, such as may be present in industrial effluents, since the elimination capacity is limited by the toxicity of degradation products.

So far, no aerobic transformation has been found with perchlorinated compounds, although there seems to be no reason why this is impossible *per se*. Anaerobic conversions that cause dechlorination have been described for several highly chlorinated compounds such as carbon tetrachloride, perchloroethylene, and 1,1,1-trichloroethane. These conversions could become very important for removing chlorinated aliphatics at low redox potential, for example, in anaerobic subsurface environments. An interesting option might be to combine anaerobic treatment steps for initial dechlorination of highly chlorinated compounds such as perchloroethylene to TCE and dichloroethylenes, with oxidative treatment to give complete mineralization.