Summary and conclusions

Biodegradation of haloaliphatic compounds

The production and use of short chain halogenated aliphatic compounds imposes problems on the environment. Most such compounds are not or incompletely degraded because enzyme activities neccessary for their conversion are absent or toxic intermediates inhibit further breakdown. The poorly degradable compounds will remain in the biosphere and represent a potential threat to all living organisms because of their toxicity and suspected carcinogenicity. Nevertheless, some halogenated aliphatic compounds can be degraded by microorganisms.

1,2-Dichloroethane (DCE) is produced in larger amounts than any other halogenated chemical and is mainly used for the synthesis of vinylchloride. DCE is not known to occur naturally and is not readily degraded in the environment. *Xanthobacter autotrophicus* GJ10 can use DCE as sole source of carbon and energy and was isolated from a mixture of activated sludges from a wastewater treatment plant and soil samples from various chemically polluted sites in the province of Groningen (Janssen et al, 1984). The first step in degradation of DCE is the hydrolytic dehalogenation to 2-chloroethanol by a haloalkane dehalogenase. This intermediate is oxidized to chloroacetaldehyde by a periplasmic alcohol dehydrogenase. Chloroacetaldehyde is further oxidized by a haloacid dehalogenase to chloroacetate which is dehalogenated to yield glycolate.

The goal of the research described in this thesis was to obtain more insight in the biochemical pathway of 1,2-dichloroethane degradation and the evolution of 1,2-dichloroethane degrading bacteria. For this, enzymes were characterized and mechanisms that lead to genetic adaptation towards degradation of these compounds were studied.

Genetics and biochemistry of haloacid dehalogenase

The purification and characterization of the haloacid dehalogenase from an overexpressing mutant of GJ10 is described in chapter 2 of this thesis. The enzyme is active with short chain α-halocarboxylic acids and acts stereospecifically on 2-chloropropionic acid: only the L-isomer is converted, with lactate being formed under inversion of configuration. The nucleotide sequence of the *dhlB* gene was determined. The protein encoded by *dhlB* shows sequence similarity to a group of L-2-chloropropionic acid dehalogenases. The catalytic mechanism of the enzyme has not yet been elucidated. Some information regarding the mechanism can be derived from sequence alignments with related proteins. It has been shown that DhlB shares three common residues with the members of a large family of hydrolytic enzymes (Koonin and Tatusov, 1994). These conserved residues might constitute the active site of DhlB.

Although the expression of *dhlB* appears to be constitutive, higher amounts of the enzyme are produced in the stationary growth phase. To obtain more insight in the regulation of its expression, the nucleotide sequence of the region upstream of *dhlB* was determined (chapter 3). Together with the results of expression experiments, it is concluded that there are two additional genes, *dhlC* and *dhlR*, located in this region that could be involved in the metabolism of haloacids. Sequence comparisons suggest that these substrates may be transported to the cytoplasm by the gene product of *dhlC*. The expression of *dhlC* and *dhlB* is probably dependent on the protein encoded by *dhlR*, the sequence of which shows similarity to a large family of proteins which activate transcription from a -24/-12 promoter together with σ^{54}. Such a promoter sequence was found upstream of *dhlC*. Additional genetic

and biochemical studies are required to confirm that *dhlC* and *dhlR* are necessary in the degradation of haloacids.

Genetic adaptation to halogenated aliphatic compounds

Chloroacetaldehyde is a reactive and potential critical intermediate in the degradation of DCE. The role of aldehyde dehydrogenases was therefore studied in chapter 4. In one of the chloroacetaldehyde negative mutants of *X. autotrophicus* GJ10, haloalkane dehalogenase activity and resistance to $HgCl_2$ was also lost. It was concluded therefore that this mutant had lost the plasmid pXAU1 (Tardif et al, 1991). Although there is also a chromosomally encoded aldehyde dehydrogenase, the plasmid encoded chloroacetaldehyde dehydrogenase is essential for degradation of DCE and 2-chloroethanol. This enzyme is the most abundant protein in extracts of GJ10 grown with DCE. The rather low affinity of the purified chloroacetaldehyde dehydrogenase for chloroacetaldehyde may explain that a high expression is neccessary to prevent toxic concentrations of this compound in the cytoplasm.

Chapter 5 describes the genetic adaptation of *X. autotrophicus* GJ10 to toxic concentrations of bromoacetate. The resistance of mutants to this substrate was accompanied by a higher haloacid dehalogenase activity. This indicates that the toxic effect of bromoacetate can be overcome when its intracellular concentration is decreased. In one of the mutants, dehalogenase overexpression appears to be the result of the insertion of a DNA fragment immediately upstream of the *dhlB* gene. This DNA fragment had the characteristics of an insertion element. Mobilization of the *dhlB* gene together with this insertion element to a plasmid could even further increase the expression of haloacid dehalogenase and the resistance to bromoacetate.

Increase in enzyme activity was also observed after adaptation to 2-bromoethanol of the 2-chloroethanol utilizing *Pseudomonas* sp. GJ1 (chapter 6). In the mutant, the levels of the NAD-dependent chloroacetaldehyde dehydrogenase were higher, thus accounting for more than half of the total soluble protein in the cell. As in the case of the enzyme from *X. autotrophicus* GJ10, the affinity of the purified chloroacetaldehyde dehydrogenase for both chloroacetaldehyde and bromoacetaldehyde was low.

X. autotrophicus strains GJ10 and GJ11 and *Ancylobacter acquaticus* strains AD20, AD25, AD27 use the same pathway for DCE degradation (van den Wijngaard et al, 1992). Plasmids similar to pXAU1 from *X. autotrophicus* GJ10 were identified in the other DCE-degrading strains (chapter 7). The *dhlA* genes of these organisms were found to be plasmid encoded. The region surrounding *dhlA* appears to be strongly conserved, since in all DCE-degrading organisms, *dhlA* is located on identical restriction fragments. The genes encoding haloacid dehalogenase are located on different restriction fragments, although the genetic organization of the *dhlB* region may be similar in some of the strains. The presence of different haloacid dehalogenases in DCE degrading strains was confirmed by activity staining on native gels and by Western blotting. Thus, it appears that the haloacid dehalogenases have diverged much further than the haloalkane dehalogenase.

Concluding remarks

The studies described in this thesis and the results of Pries *et al.* (1994) have shown that different genetic mechanisms are involved in the adaptation towards utilization of halogenated aliphatic compounds. Mutations in dehalogenases may be responsible for an increased or altered range of substrates (Pries *et al.*, 1994). Insertion elements can activate the expression of catabolic enzymes and may be important in the recruitment of genes from the chromosome to plasmids. Finally, plasmids

harbouring dehalogenases and other catabolic enzymes may be transferred to other bacterial genera, causing an increased diversity of combination of genotypes.

The evolution of bacteria which are able to grow with 1,2-dichloroethane is presumably the result of several adaptational events. We propose that, starting from an organism capable of growing with glycolate, the acquisition of a haloacid dehalogenase gene or the adaptation of a preexisting hydrolytic enzyme to an enzyme that hydrolyzes haloacids could have enabled this organism to grow with chloroacetate. Haloacetates do occur naturally, and it is likely that organisms able to grow with these compounds have been present before DCE was introduced in the environment. A second evolutionary step, could have been the modification or increased expression of an aldehyde dehydrogenase, which results in a decreased intracellular level of toxic chloroacetaldehyde. There are no indications that the alcohol dehydrogenase is specifically adapted towards the oxidation of haloalcohols. The recruitment of a haloalkane dehalogenase therefore would allow the organism to grow with DCE. The second and third adaptational events have probably ocurred after introduction of DCE in the environment.

Many short chain halogenated aliphatic compounds remain recalcitrant to biodegradation. It may well be possible in the future to construct or adapt bacteria to degrade these compounds by using in vivo selection, combining catabolic enzymes and protein engineering.