Summary and general conclusion

This thesis describes physiological characteristics of *Pseudomonas oleovorans* during growth in a two-liquid phase medium which contains a bulk organic phase.

*P. oleovorans* is a metabolically versatile *Pseudomonas* strain, which contains an alkane hydroxylase system that is able to oxidize a wide range of hydrocarbons. Some of the oxidation intermediates are useful for the production of fine chemicals. Studies of this bacterium and its alkane hydroxylase system began more than 30 years ago, and since then, the genetics and enzymology of the alkane hydroxylase system have been studied intensively. Meanwhile, application processes of the alkane hydroxylase system are being developed and optimized.

Since most hydrocarbons are poorly soluble in aqueous media, the transformation of these compounds by microorganisms are generally carried out in two-liquid phase systems which contain organic solvents as a second phase. One of the major drawbacks in the application of microorganisms in two-liquid phase media is solvent toxicity to the cells. Many organic solvents are antimicrobial agents. In the presence of toxic organic solvents, the cells easily lose their viability and enzyme activity, leading to lower productivity and production rates.

To improve biotransformation productivity, it is important to understand the effects of toxic organic solvents on cells as well as the resistance mechanisms of cells to solvents. Based on this information, it might be possible to protect cells during biotransformation processes, leading to higher productivity. A basic aim of this thesis is to gain more insight in the effects of solvents on bacteria.

Initial studies of the effects of organic solvents on *P. oleovorans* were performed during growth of the cells on octane or citrate in continuous cultures, in which steady state conditions were attained (Chapter 2). Since the cell membranes are the primary site of action of toxic organic solvents, we focused on the effects of organic solvents on the membrane composition and properties at various growth rates. Here, we measured not only the membrane lipid fatty acid composition, but also the transition temperature of the membrane lipids. Compared to citrate-grown cells, we found that in response to growth on octane, the cells changed their fatty acid composition. As a consequence of these changes, the membrane lipid phase transition temperature increased from 6°C to 24-30°C as shown by differential scanning calorimetry measurements. Such a change of the membrane fatty acid composition is expected to compensate for a decrease in membrane phospholipid order during growth on octane. A striking result obtained in these experiments was that in addition to changes of mean acyl chain length and the ratio of saturated fatty acids to unsaturated fatty acids, *P. oleovorans* used an additional strategy, namely cis-to
trans unsaturated fatty acid isomerization, to regulate its membrane lipid fluidity.

When *P. oleovorans* was grown on octane, two different processes occurred. First, the cell membranes were exposed to a bulk apolar octane phase. Second, the alk genes were induced in order to use octane as carbon source. To further distinguish the effects of these two processes on the fatty acid composition, we have compared the effects of growth in (or on) octane and of inducing the alk genes on the fatty acid composition of three types of *P. oleovorans* strains (Chapter 3). We found that octane itself caused no changes in the fatty acid composition if the alk genes were absent, or the alk genes were not induced, or the alk genes were inactivated. The changes of fatty acid composition during growth on octane were triggered by induction of the alk genes with octane. An increase of mean acyl chain length due to an increasing amount of fatty acid 18:1 was shown to relate to the synthesis of AlkB, a major integral membrane protein. This suggested that insertion of a large amount of AlkB into the membrane could directly or indirectly affect activities of enzymes involved in fatty acid and phospholipid synthesis. The formation of trans unsaturated fatty acids resulted mainly from the effects of 1-octanol, an octane oxidation product. By converting cis-unsaturated fatty acids to their trans isomers, the cells can decrease the membrane fluidity and thereby compensate the increase of membrane fluidity engendered by l-octanol. Based on these results, a simple model was proposed. However, to gain an insight into these processes in molecular detail, more research will be needed.

As a by-product of studies described in chapter 3, we showed in Chapter 4 that induction of the alk genes had deleterious effects on the cells. Following induction and expression of the alk genes, there was a reduction of growth rate and formation of filaments. The stress which alk induction imposed on the cells resulted in loss of alkane oxidation activity both in the wild type strain GPol and various recombinant strains when these strains were grown under non-selective conditions. Although the observed effects were correlated with the expression of AlkB, our data suggested that AlkB is not the sole alk gene product which has these effects. What else contributes to these negative effects remains to be determined. Understanding how bacteria sense and inactivate undesired gene expression might enable us to develop strategies to circumvent these effects for the production of stable biocatalysts.

Isomerization of cis-unsaturated fatty acids to their trans isomers is a special regulation system of membrane fluidity found in *Vibrio* sp. and *Pseudomonas putida* (e. g. *P. oleovorans*). In other bacteria, no such regulation system has yet been found.
Summary and general conclusion

By investigation of in vivo and in vitro cis/trans isomerization in the presence or absence of alkanes and alkanols (Chapter 5), we learned how P. oleovorans uses this system to regulate its membrane fluidity in response to toxic organic solvents. Our results showed that the cis/trans isomerization is catalyzed by a constitutive periplasmic isomerase and is independent of de novo fatty acid synthesis. We found that the toxic organic solvents triggered the cis/trans isomerization by effects on the structure and the physical state of the membrane rather than by activation of the isomerase. Accumulation of the toxic organic solvents in the membrane resulted in expansion and fluidization of the membrane, which allowed the periplasmic isomerase to access fatty acid double bonds and to change the configuration of the double bonds from cis to trans.

Given these results, we expect that the isomerisation will be facilitated and the cis/trans isomerization will occur not only as a result of exposure of cells to toxic organic solvents, but also by other mechanical or physical processes, which can alter the membrane structure to an extent that fatty acid double bonds become accessible. Moreover, the properties of the cis/trans isomerization indicate that it is a rapid and energetically inexpensive system with which to modulate membrane lipid fluidity. This regulation system might be especially important to allow cells to adjust their membrane fluidity in response to toxic organic solvents when neither the ratio of unsaturated to saturated fatty acids nor the mean acyl chain length of the membrane lipids can be changed sufficiently to attain the desired change of membrane fluidity, due to a decrease of phospholipid and protein synthesis caused by inhibition of these processes by the toxic organic solvents. The availability of a cis/trans isomerase might well explain why Pseudomonas shows the highest solvent-tolerance among all microorganisms tested so far. However, to further clarify the mechanism of solvent resistance in bacteria, it is necessary to further investigate the role of the isomerase. Future experiments may include determination of the N-terminal amino acid sequence of the isomerase, cloning of relevant genes, purification of the isomerase, and determination of the isomerase structure.

Similar to the long standing debate on the mechanism of general anesthetic effects of organic solvents, it is still unclear whether proteins or lipids are the primary site of solvent toxicity during exposure of microorganism to toxic organic solvents. Furthermore, discovery of efflux systems that actively transport hydrophobic antimicrobial compounds to the extracellular environment suggests that specific proteins might also play an important role in resistance of bacteria to toxic organic solvents. Accordingly, it is necessary to extend our study from membrane lipids to
proteins. Comparison of protein profiles of cells grown in the presence or absence of organic solvents will be the first step to approach this issue. We initiated such a study by setting up a two dimensional gel electrophoretic system for analysis of Alk proteins which are expected to be present during growth on octane (Chapter 6). With Coomassie staining, 6 of the Alk proteins were assigned in two dimensional gel electrophoresis maps. Analysis of proteins from different cell fractions showed that AlkG was strongly associated with the membrane through AlkB. However, further technical improvement will be necessary in order to detect protein patterns of cells in response to growth in the presence of toxic organic solvents.

In conclusion, the growing interest in the application of microorganisms in biotransformation in two-liquid phase system has stimulated the investigation of physiological responses of microorganisms to toxic organic solvents. Data on the effect of toxic organic solvents on cells are being rapidly accumulated, and an increasing number of more solvent-tolerant strains has been isolated. However, many questions remain to be answered. Understanding solvent toxicity and the resistance of microorganisms to toxic solvents in molecular detail is a future task in this field.