Towards rational strain improvement?

The high-yielding *Penicillium chrysogenum* strains used today in large-scale penicillin production are the outcome of over 50 years of random mutagenesis, screening and selection. Even at present, better producing strains are still obtained through empirical methods such as random mutation by UV-radiation and the use of selective pressures, e.g. addition of metabolic inhibitors or intermediates of primary metabolism. Although this classical approach of strain improvement is in itself a powerful procedure, it is severely limited by a moderate mutagenic capacity and lack of specificity. In time these limitations will make it more difficult to obtain higher-yielding strains through conventional strain improvement procedures.

During the last two decades, biochemistry and genetics of β-lactam antibiotics advanced rapidly and tools for recombinant DNA technology in *P. chrysogenum* have become available. These developments make a more direct and rational approach to strain improvement feasible: metabolic engineering. Metabolic engineering implies the combined improvement of strains through genetic engineering and tuning of production processes. This approach could boost yields of production strains beyond limits that are difficult or even impossible to overcome by classical strain improvement procedures. However, successful and prolonged application of metabolic engineering demands amongst others an accurate stoichiometric model, proper tools to determine the basic biochemical flows, substantial genetic know-how and detailed knowledge of
the involved biosynthetic pathways. These basic conditions have lead to scepticism about the applicability of metabolic engineering in improvement of industrial *P. chrysogenum* strains in the near future. It is often argued that the complexity of the mycelium (a non-stationary multi-cellular system with a complex growth-mode and containing a diversity of intra-cellular compartments) and the lack of proper tools to determine biochemical flows accurately will make it difficult to obtain all the data needed for the construction and completion of a practical model.

Improvement of strains will in the near future probably proceed through both conventional methods and metabolic engineering, the latter mainly based on common sense and intuition and less often on thorough analysis. Nonetheless, this approach still demands substantial genetic and biochemical know-how and a detailed insight in the physiology of penicillin biosynthesis. Solid information on genetics, biochemistry and physiology of filamentous fungi does not only benefit industrial research: it is of fundamental importance as well. Extensive research at penicillin biosynthesis will enhance our perception of the general and specific principles underlying the formation of secondary metabolites in fungi and it will contribute to a better understanding of the physiology of filamentous fungi in general.

**Are transport processes potential bottlenecks in penicillin production?**

Considering metabolic engineering of industrial strains raises also the question which role transport processes play in penicillin biosynthesis. Although the obtained final penicillin titers are high in current industrial production processes, the average rate at which penicillin is formed during the production stage is low. High maximum rates of nutrient uptake and product secretion are therefore not needed to sustain penicillin production. Hence, when supplied at sufficient levels uptake of nutrients is unlikely to form a bottleneck in penicillin production (e.g. the rate of phenylacetic acid uptake is substantially higher than the rate of penicillin production, chapter 5 of this thesis). In contrast, the secretion of penicillin could be a critical process. Since the final step in penicillin biosynthesis comprises the conversion of a hydrophilic penicillin into a more hydrophobic one, release through simple diffusion seems the most plausible mode of secretion. However, this is not a conceivable mechanism in industrial strains. The slow rate at which penicillin is produced in industrial production processes does not exclude passive diffusion per se, but the high final titers (at least 125 mM) are indicative of an active process. Our studies with model systems showed that the penicillins G and V diffuse rapidly across simple lipid bilayers and are able to dissipate a transmembrane pH-gradient. Penicillins are therefore toxic to living cells in particular at the high concentrations encountered at the end of the production stage.

Based on the effect of ergosterol incorporation on penicillin permeability of
model membranes, we anticipate that the presence of 20 mol% ergosterol endows the
*P. chrysogenum* plasma membrane with a low permeability to penicillins. Preliminary
experiments using mycelial suspensions and protoplasts of a low producing strain
showed that penicillin G is effectively excluded, indicative of the presence of an active
secretion system [Hillenga, 1994, unpublished results]. Most transport systems capable
of translocating β-lactam antibiotics identified thus far transport hydrophilic β-lactams.
Recently, Nikaido and colleagues [212] showed that the *Salmonella typhimurium*
AcrAB efflux pump transfers resistance to β-lactam antibiotics in the complete absence
of β-lactamase. Especially β-lactams with lipophilic side-chains showed to be good
substrates for the AcrAB pump. It is therefore not unlikely that a similar transporter
belonging to one of the multidrug resistance transporter families is involved in
secretion of moderately hydrophobic penicillins in *P. chrysogenum*. The detection of
this putative penicillin transporter might however prove to be difficult. Next to
transport studies in mycelium and less complicated systems a genetic approach seems
the best way to tackle this problem. Although elucidation of the mode of penicillin
excretion has to be a main topic in future research, nutrient uptake and other transport
processes should not be ignored. Information on the location and levels of transport
systems and their characteristics (e.g. specificity) will be valuable in constructing an
accurate stoichiometric model for metabolic engineering or when considering the use
of novel nutrients or the design of new pathways.

**The complications of physiological studies on penicillin biosynthesis.**

Physiological studies in filamentous fungi are severely hampered by their
complex growth characteristics and miscellaneous cellular and sub-cellular
morphology. Correct assessment of data such as localization and concentration of
solute and enzymes, internal pH values and transmembrane gradients, is difficult or
even impossible with the methods currently available. Studies on penicillin
biosynthesis in *P. chrysogenum* are additionally frustrated by the following: i)
penicillin formation presumably only takes place in the non-growing metabolically
active hyphal regions; ii) penicillin biosynthesis proceeds in different cellular
compartments. Several of these obstacles can be circumvented by the use of less
complicated models like the system described in this thesis or by using isolated “intact”
organelles or their membranes.

Membrane-located processes like transport can be studied in great detail in
simple model systems. The studies presented in this thesis showed that a model
membrane system based on isolated plasma membranes can be used to elucidate the
transport mechanism of various solutes. Features such as kinetics, identity of the
coupling ions, stoichiometry and specificity can be determined very accurately.
However, the simplicity of the model system also limits its applicability and the extrapolation of obtained data. In intact mycelium different ion gradients are sustained concurrently across the plasma membrane and transport of solutes interacts with many physiological processes. Moreover, the cellular differentiation and distinct way of growth of filamentous fungi has resulted in specific adaptations of the plasma membrane. Of these the two most typical ones are: i, the irregular distribution of the H\(^+\)-ATPase and nutrient carriers along the hyphae; and ii, the presence of a current and pH/ion gradients along hypha. The model system we developed is not suitable for studies on these and several other features of plasma membrane located processes and their interactions with intracellular processes. A miscellaneous approach might give more insight, e.g. using antibodies against the plasma membrane ATPase and solute transporters, labeling of these enzymes with fluorescent substrates and micro-electrode measurements.

**Future studies on nutrient uptake in penicillin biosynthesis.**

Interesting topics for future studies in the developed model system are amongst others: transport of sugars (e.g. glucose and lactose), uptake of inorganic phosphate and the regulation of sulfate transport. At present the mechanism of glucose and lactose transport is unclear, active transport or facilitated diffusion are possible mechanisms. In case of active transport, probably one mole of ATP is consumed per mole of glucose imported, while facilitated diffusion does not demand the input of ATP. Studies on glucose consumption in C-limited continuous cultures of *P. chrysogenum* showed an affinity of 0.02 mM, indirectly suggesting the presence of a high-affinity transporter. Preliminary studies on sugar transport in *P. chrysogenum*, demonstrated that protonophores instantly inhibit the uptake of both glucose and lactose in mycelial suspensions (Hillenga 1994, data not shown). This observation seems to support the presence of active transport systems for these sugars. However, it is well known that addition of protonophores strongly affects the overall energetic state of the cell. Upon addition of protonophores, cytosolic ATP levels decline within seconds and ATP consuming processes such as the phosphorylation of sugars are severely effected. Hence, uptake of mono- and disaccharides in *P. chrysogenum* might take place through active transport but uptake through facilitated diffusion can not be excluded at present. Proton-motive-force (pmf) driven uptake of glucose or lactose in hybrid plasma membrane vesicles could not be demonstrated so far.

In *Neurospora crassa* indications have been obtained that inorganic phosphate is taken up in symport with sodium ions. Since in the studies described in this Thesis we only demonstrated pmf-driven transport, solid evidence for solute uptake driven by a sodium gradient would show that filamentous fungi are able to utilize different ion
gradients for solute uptake. Studies at inorganic phosphate transport in *P. chrysogenum* might therefore be very interesting, especially from a fundamental point of view. The conversion of sulphate to L-cysteine is one of the primary metabolic routes through which the flux must have increased significantly due to strain improvement. Uptake of sulfate is strictly regulated and subject to genetic and metabolic control mechanisms. These control mechanisms might be severely altered in industrial strains resulting in elevated uptake of sulphate during penicillin production. In addition to the transport studies with the developed model system, a genetic approach is needed to identify and characterize the mechanisms involved.

**Additional topics**

Our studies showed that ergosterol plays a major role in the penicillin permeability of the *P. chrysogenum* plasma membrane. The biosynthesis of ergosterol seems therefore an attractive target in strain improvement and it is feasible that the ergosterol content or the sterol composition of the plasma membrane has been altered due to the applied mutagenic procedures. Since information on industrial strains is classified, it is unknown whether plasma membranes of high producing industrial strains contain more or less ergosterol than plasma membranes of low producing strains. When indications are obtained for a specific tendency, this information could possibly be used in strain improvement. Moreover, a specific trend might indicate which mechanism of penicillin excretion is operative in industrial strains. Comparison of the overall composition of plasma membranes from strains that produce different levels of penicillin possibly reveals other differences.

As discussed in chapter 1 of this thesis, the microbody plays a central role in penicillin biosynthesis. Studies at the secretion of penicillin and the role of transport in penicillin biosynthesis should not be limited to the plasma membrane but also involve this organelle. Fluxes through the microbody have to be high to prevent the accumulation of several products inside the microbody. How the produced hydrophobic penicillin and other compounds leave the microbody is unclear. When given the opportunity, hydrophobic penicillins like penicillin G and V will certainly insert into intracellular membranes and pollute organelles. To prevent this, *P. chrysogenum* has to sustain low cytosolic levels of the hydrophobic penicillin produced. This can be achieved through effective removal from the cell via a plasma membrane located transport system. The penicillin formed has to be contained in a specific compartment prior to release, e.g. secretory vesicles. Penicillin could leave the microbody through a specific transport system or through diffusion via aspecific porins. Homogenous penicillin microbody fractions suitable for *in vitro* studies have not been isolated today. Consequently, no information is available on the physiology of this *P. chrysogenum*
In conclusion

“What is so interesting about transport processes in P. chrysogenum”? “Over forty years of research, everything must be known by now, the field you are working in seems not that interesting to me”. These type of questions and remarks were frequently prompted after presentations on the research described in this thesis. In short, to “outsiders” research at penicillin biosynthesis has the image of being outdated, and mainly based on commercial motives. That the commercial value of penicillin is often one of the reasons to initiate studies on P. chrysogenum is true. However, there are also important basic scientific motives to examine penicillin biosynthesis in this fungus. Despite many years of research, our knowledge on several aspects of penicillin biosynthesis is rather poor. Nonetheless, due to the fast and still rapidly growing knowledge on penicillin biosynthesis and its regulation, P. chrysogenum is regarded a model organism in fungal secondary metabolite formation. From a genetic point of view, Aspergillus nidulans is more suitable to study specific features of penicillin biosynthesis. But the wealth of public information and especially the availability of high-producing strains make P.chrysogenum the organism of choice for research on transport processes. The studies described in this thesis should be regarded as a first step towards elucidation of the role of transport processes in β-lactam formation. Future research at plasma membrane located transport processes and at the microbody membrane should reveal for example how penicillin is secreted and if next to a proton circuit other ion circuits play an important role in nutrient uptake across the plasma membrane. The developed model system can be a valuable tool in these studies but the use of more complex systems should not be avoided.