Protein export in bacillus subtilis and escherichia coli

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SUMMARY AND GENERAL DISCUSSION

The export of heterologous proteins in *Bacillus subtilis* and *Escherichia coli* is often inefficient. Frequently observed problems are: 1), accumulation of the precursor form of the exported protein in the cytoplasm or in the membrane; 2), inefficient or incorrect processing of the precursor; 3), inefficient release of the processed protein from the cell-envelope; and 4), proteolytic degradation of the exported protein. Several distinct steps in the export of proteins, involving properties of both the exported protein and the cellular protein export apparatus, can be conceived to account for these problems. The main purpose of the investigations described in this Thesis was to determine whether the processing of precursors of, in particular heterologous, exported proteins by signal peptidase I (SPase I) might be a rate-limiting step in protein export.

In chapter I a general introduction in bacterial protein export processes is presented. Particular properties of exported proteins, components of the cellular protein export machineries of *Escherichia coli* and *Bacillus subtilis* and energy requirements for protein export are summarized. These properties are discussed in relation to problems encountered in the export of heterologous proteins by bacteria.

Chapter II describes the construction of a mutant of *Escherichia coli* (N4156::pGD28) in which SPase I synthesis could be controlled by a temperature-sensitive repressor. Repressed SPase I synthesis resulted in a severe inhibition of growth. Using this mutant, the effects of SPase I limitation on the synthesis and efficiency of processing of *E.coli* TEM-β-lactamase and *Bacillus licheniformis* α-amylase were studied. SPase I limitation resulted in reduced rates of processing of pre-β-lactamase and in a strong inhibition of the synthesis of α-amylase. The data indicated that reduced levels of SPase I limited the efficient processing of pre-β-lactamase and the synthesis of α-amylase in *E.coli*.

Chapter III describes the use of *E.coli* N4156::pGD28 (see chapter II) for the selection of the *Salmonella typhimurium lep* gene, encoding SPase I. The nucleotide sequence of the *S.typhimurium lep* gene was determined. This gene encoded a protein of 324 amino acids, showing 92.6% identity with the *E.coli* SPase I. Expression of the gene in *E.coli* N4156::pGD28 resulted in the suppression of growth inhibition and in the restoration of

processing activity on TEM-β-lactamase under conditions of *E.coli* SPase I limitation. The results suggested that the system described here has the potential to be used for the cloning and expression of other heterologous SPase I-encoding genes.

Chapter IV describes attempts to clone the *B.licheniformis lep* gene. Fragments of the *E.coli* and *S.typhimurium lep* genes were used as hybridization probes for Southern blotting. Two hybridizing fragments were obtained. However, introduction of either of fragment in *E.coli* N4156::pGD28 could not complement defective SPase I production in this mutant (data not shown). Moreover, DNA sequence analysis indicated that they did not contain *lep* genes. Instead, the proteins encoded by these fragments showed similarity with a higly conserved ATP-binding component of binding protein-dependent transport systems and with L-asparaginases, respectively. The absence of sequence similarity suggests that differences exist between the *lep* genes of *E.coli*/*S.typhimurium* and *B.licheniformis*. The "false" positive signals in the Southern hybridizations were probably due to several adjacent sequences on the *B.licheniformis* DNA fragments acting cooperatively in the creation of a hybridization signal. A third *B.licheniformis* DNA fragment hybridized with an *E.coli* DNA probe containing not only *lep* sequences, but also part of the flanking *lepA* gene, which has no apparent role in protein export. This fragment encoded the 3' end of the *B.licheniformis lepA* gene. Unlike the situation in *E.coli* and *S.typhimurium*, the sequences downstream of this *lepA* gene did not appear to encode proteins showing similarity with *E.coli* or *S.typhimurium* SPase I. This indicates that the *B.licheniformis lepA* and *lep* genes are not organised in an operon-like structure as observed in *E.coli* and *S.typhimurium*.

Chapter V describes the effects of overproduction of SPase I in *E.coli* on the processing kinetics of various hybrid TEM-β-lactamase precursors which contained signal peptides selected from the *Bacillus subtilis* chromosome. Two precursors (pre[A42]-β-lactamase and pre[A2d]-β-lactamase), which were processed slowly under standard conditions, showed enhanced processsing rates when SPase I was overproduced. The increase in the rate of processing was most drastic with pre(A2d)-β-lactamase. Moreover, the efficiency of release of mature (A2d)-β-lactamase into the periplasm was increased concomitantly. A third hybrid precursor (pre[A13i]-β-lactamase), which was not detectably processed under standard conditions, was processed to a limited extent under

conditions of SPase I overproduction. The mature (A13i)-β-lactamase did not appear to be efficiently released into the periplasm. In contrast, the processing rates of wild-type pre-β-lactamase and pre(A2)-β-lactamase, already being high under standard conditions, were not detectably altered by SPase I overproduction. These results demonstrate that the availability of SPase I can be a limiting factor in protein export in *E.coli*, in particular with respect to (hybrid) precursor proteins showing low (SPase I) processing efficiencies. At present we can only speculate about the mechanisms which cause increased processing efficiencies of pre(A2d)- and pre(A13i)-β-lactamase under conditions of SPase I overproduction. It is conceivable that these hybrid precursors are only processing-competent during a limited period of time and that increased availability of SPase I allows the processing of a larger fraction of processing-competent molecules before these become a poor substrate.

Chapter VI describes experiments on the production of *E.coli* SPase I in *B.subtilis*. By analogy to the results described in chapter V, it was reasoned that the overproduction of SPase I might also result in improved secretion of hybrid precursor proteins in *B.subtilis*. Since no *lep* genes of Gram-positive bacteria were available, the approach chosen to address this question was to express the *E.coli lep* gene in *B.subtilis*. To that purpose the *E.coli lep* gene was provided with *B.subtilis* expression signals and cloned on a multi-copy-number plasmid (pGDL24) in this organism. This resulted in approximately 2.5-fold higher amounts of SPase I per mg cellular protein as compared to wild-type *E.coli*. However, pulse-chase labeling studies revealed that the *E.coli* SPase I in *B.subtilis* did not result in increased rates of processing of two hybrid secretory precursors (pre[A13]-α-amylase and pre[A2d]-β-lactamase). As suggested by *in vitro* processing studies, the failure to achieve increased rates of processing may be due to inactivity of the *E.coli* SPase I when produced in *B.subtilis*. Moreover, unlike in *E.coli*, the enzyme did not appear to be exposed on the outside of the cytoplasmic membrane. This suggests that the *E.coli* SPase I was not inserted correctly into the *B.subtilis* cytoplasmic membrane.

In summary, the results presented in this Thesis indicate that the availability of sufficient quantities of SPase I is critical for efficient export of proteins into the periplasm of *E.coli* (chapters II and V). The observation that the efficiency of protein export could be improved by overproduction of SPase I in *E.coli* may be of considerable practical value

(chapter V). It is conceivable that analogous approaches may be successful in attempts to improve the export of heterologous proteins by bacteria in general. However, the observation that the *E.coli* SPase I was inactive when expressed in *B.subtilis* (chapter VI), may indicate that such improvements require SPase I from the production organism itself, or from closely related species. A hybridization approach for the selection of *lep* genes, using the *E.coli lep* gene as a probe, appeared to be suitable for the cloning of the corresponding gene of the Gram-negative bacterium *S.typhimurium* (chapter III), but not for that of the Gram-positive bacterium *B.licheniformis* (chapter IV). In addition to the apparent lack of sequence similarity between the *lep* genes, two other observations suggest that the SPases of Gram-negative and Gram-positive bacteria are different. First, conditions favouring the *in vitro* processing of precursors by *E.coli* SPase I, present in cell-free extracts, did not favour the processing by the analogous enzyme, present in *B.subtilis* cell-free extracts. Second, antibodies directed against *E.coli* SPase I did not cross-react with *B.subtilis* membrane proteins, which presumably include the SPase I protein (chapter VI).