CHAPTER 8

SUMMARY AND GENERAL DISCUSSION
**SUMMARY**

*Bacillus subtilis* is the best-characterised and genetically most amenable Gram-positive soil bacterium. *B. subtilis* naturally secretes large quantities of proteins directly into the growth medium and is proven to be safe, which makes this bacterium a prime candidate for heterologous protein production. Proteins of bacilli and other Gram-positive bacteria are very well produced and secreted by *B. subtilis* and closely related *Bacillus* species. In this thesis the use of the *Bacillus subtilis* “Cell Factory” for production of pharmaceutically interesting recombinant proteins is further investigated. The research described focused on (1) the production of different model proteins (α-amylase, lipase, human interleukin-3 (hIL-3) and mutants thereof, and human TRAIL); (2) studies to evaluate the potential use of the secretion stress response of *B. subtilis* as an indicator for protein production levels, and (3) on mutagenesis of a key residue in bioactivity of hIL-3.

In chapter 3, the optimisation of production and secretion of hIL-3, a haematopoietic progenitor cell stimulating cytokine, is described. A host-vector system was developed on the basis of the multiple protease-deficient *B. subtilis* strains WB700 and WB800. By using a multicopy plasmid containing two tandemly positioned strong promoters plus an efficient signal sequence, the hIL-3 protein was efficiently produced and secreted into the growth medium to levels up to 100 mg/l. Mass spectrometry and cross-linking experiments with a thiol-specific reagent demonstrated that intact and properly folded hIL-3 was purified from the *B. subtilis* growth medium. Bioactivity tests showed that the isolated hIL-3 was able to specifically induce proliferation of the hIL-3-dependent leukaemia cell line MO7e.

The studies described in chapter 4 were aimed at the development and production of a hIL-3 variant with (ant)agonistic activity. The glutamate residue at position 22 in hIL-3 was investigated by mutational analysis, as a homologous glutamate residue in human interleukin-5 (hIL-5) and human granulocyte macrophage colony stimulating factor (hGM-CSF) was shown to be important for bioactivity. The *B. subtilis* expression system that was proven to be functional for hIL-3 production in the studies described in chapter 3 was used to obtain sufficient levels of all 19 hIL-3E22 variants. Using the hIL-3-dependent leukaemia cell lines MO7e and TF1, almost all mutants at position E22 were shown to have lost bioactivity. Interestingly, most of these mutants maintained their binding to the hIL-3 receptor α-subunit as was shown by surface plasmon resonance experiments. The virtually inactive hIL-3E22N has a
strongly reduced affinity towards the hIL-3 receptor β-subunit whereas binding to the α-subunit is with an affinity similar to wild-type hIL-3. Initial experiments show that hIL-3E22N and hIL3-E22F have antagonistic properties. Using computational design, it should be possible to choose additional positions for further mutagenesis rounds to find a strong antagonist for use in the clinic.

In chapter 5 and 6 studies are presented that were aimed at answering two important questions on protein translocation in *B. subtilis*. The first question is whether the secretion stress response is only triggered by α-amylase translocated across the membrane and the second question is whether the CssRS-dependent response is a general protein secretion stress response that can be elicited by proteins other than α-amylase. Using an α-amylase variant that is not translocated across the membrane, it was shown that non-translocated AmyQ does not provoke a protein secretion stress response. Secondly, it was demonstrated that the CssRS-dependent response is a general secretion stress response that can be triggered both by homologous (lipase) and heterologous (hIL-3) secretory proteins. However, the intensity of the protein secretion stress response was found to reflect the production levels of the respective proteins only partly. Importantly, degradation of hIL-3 by extracellular proteases has a major impact on the production level, but only a minor impact on the intensity of the secretion stress response. Other parameters, like the dependence of secretory proteins on extracytoplasmic folding catalysts or their susceptibility to extracellular proteases also seem to determine the correlation between the secretion stress response intensity and protein production levels.

Some preliminary results on production and secretion of human TRAIL (hTRAIL; TNF Related Apoptosis Inducing Ligand) using *B. subtilis* are described in chapter 7. An attempt was made to secrete the soluble part of hTRAIL, which forms a homotrimer, using the production system as described in chapter 3. While this production system leads to production of hTRAIL and its presence in the cellular compartment was proven, only a very small amount of extracellular hTRAIL was detected. To optimise the secretion of hTRAIL, two strong promoters (P43 and nap), a range of signal peptides to target the precursor protein to the Sec or TAT secretion machinery, and different growth conditions were used. The influence of adding citrate to improve growth conditions or Zn\(^{2+}\) ions to catalyse proper folding of the hTRAIL homotrimer was investigated. It was difficult to prove that the hTRAIL found in the growth medium is actively secreted mature protein, and subcellular localisation studies of hTRAIL were not conclusive. Finally, hTRAIL semi-purified from different *B. subtilis* WB800 cellular and growth medium fractions was shown to have bioactivity towards Colo205 cancer cells.
In conclusion, the observations described in this thesis show that *B. subtilis* is an efficient production system for a subset of heterologous proteins. Once an optimised protein specific production system has been established, the system is very useful for the production of mutants of that protein. However, not all heterologous proteins are even amenable to the wide variety of approaches that can be applied for the optimisation of the cell factory. *B. subtilis* cells can show a general secretion stress as a response to high-level production and secretion of a protein.

**GENERAL DISCUSSION AND FUTURE PERSPECTIVES**

*B. subtilis* as a Cell Factory is a research subject that fascinates many researchers all over the world. Strain improvement is not only limited to the ongoing search for useful strong promoters, signal sequences, and expression vectors (Brockmeier *et al.*, 2006a and b; Phan *et al.*, 2006; Nguyen *et al.*, 2007; Fu *et al.*, 2008), but also more innovative tools are being explored such as genome minimisation (Westers *et al.*, 2003; Ara *et al.*, 2007), which recently led to an enhanced productivity of a recombinant protein (Morimoto *et al.*, 2008). In recent years it has become apparent that especially the process of secretion of a certain heterologous protein has its own demands. The hIL-3 production system that is described in this thesis might very well be usable for other four α-helix bundle cytokines, *e.g.* interleukin-5 or GM-CSF. The signal sequence seems not the only determinant for efficient secretion, also (part of) the mature protein is involved, which became apparent in our studies trying to produce hTRAIL in the production system that was optimised for the production of hIL-3. Previous studies of the bottlenecks of protein secretion in *B. subtilis* have shown that the secretion bottleneck is determined largely by the secreted protein itself and, to a lesser extent, by the combination of the signal peptide and secretory protein (Bolhuis *et al.*, 1999a). This is also reflected by studies of Brockmeier *et al.* (2006a) in which attempts to maximise the secretion of heterologous proteins using optimal signal peptides failed, indicating that there is still a lack of knowledge with respect to the complex interactions between the signal peptide and mature protein domains (Harwood and Cranenburgh, 2008).

For hIL-3, secretion by *Bacillus licheniformis* was already demonstrated. The secretion system for this protein had to be adapted for *B. subtilis*. Obtaining secretion of the trimeric protein hTRAIL turned out to be a bigger challenge and the studies described in this thesis revealed some bottlenecks that have not been overcome until now. The *B. subtilis* secretion pathway can be divided into
three functional stages: (i) the intracellular synthesis of secretory pre-proteins at the ribosome and targeting to the translocase, (ii) translocation across the cell membrane, and (iii) late stages, including the removal of the signal peptide and release from the translocase. The last step is followed by the folding of the protein in the correct conformation (in case of secretion via the major Sec machinery) and passage of the cell wall for real secretory proteins (Tjalsma et al., 2000; Li et al., 2004). From our studies it is clear that for high level secretion of hTRAIL some of these processes have to be investigated in more detail to achieve a more versatile production system using B. subtilis. Some examples of possible Bacillus subtilis Cell Factory improvements for model proteins, such as hTRAIL that are difficult to express, are listed and explained below.

In theory, it would be beneficial for the protein to stay in a non-folded conformation as long as possible, to prevent malfolding and intracellular multimerisation. Premature folding might lead to buried signal peptides, preventing secretion. Harwood and Cranenburgh (2008) describe that the rate of folding of proteins that emerge from the secretion machinery is important for minimising aggregation and misfolding. Rapid folding is facilitated by a combination of intrinsic and extrinsic factors. Different types of folding factors have been identified in B. subtilis, like intracellular (GroE and DnaK series, CsaA,) membrane bound (HtrA/B) and extracytoplasmic chaperones (peptidyl-prolyl cis-trans isomerases (like PrsA), and thiol-disulphide oxidoreductases), propeptides, and metal ions (Harwood and Cranenburgh, 2008; Fu et al., 2008). Overexpression of cytoplasmic chaperones might help the precursor protein to maintain a translocation-competent conformation. Previous studies showed that the intracellular chaperone CsaA could stimulate in vitro translocation of prePhoB and could prevent in vitro aggregation of luciferase (Müller et al., 2000a and b). However, the exact functioning of CsaA is unknown and the effect of overexpression of this protein has not been tested to our knowledge. If the production rate of a disulphide-bond containing protein is high, the disulphide bond forming capacity of the cells may become saturated, leading to the formation of non-native disulphide bonds and thus malfolding. The importance of the thiol-disulphide oxidoreductases BdbC and BdbD for the post-translocational folding of Escherichia coli PhoA in B. subtilis was shown previously (Bolhuis et al., 1999b; Miyauchi et al., 1999; Meima et al., 2002; Darmon et al., 2006). It has been proposed that BdbB, BdbC, and BdbD primarily act as oxidases (Sarvas et al., 2004). Consequently, it seems difficult to predict the effect of overexpression of these BdbS on the production of hTRAIL. Although overexpression of certain BdbS might positively influence production levels of disulphide bridge(s)-containing proteins, it could on the other hand be counter-productive for hTRAIL as the single cysteines in this
protein are uniquely liganded to a $\text{Zn}^{2+}$ ion in the trimer (Hymowitz et al., 1999 and 2000; Bodmer et al., 2000). For the secretion of the active form of hTRAIL it seems beneficial to keep the monomer shortly in a reduced state, so that disulphide bridges between cysteines are prevented, the signal peptide can be cleaved off, and the stabilising zinc ion can bind in the pocket of the trimer. Signal peptide processing has been shown to be a secretion bottleneck for those proteins that end up in the growth medium but whose precursors are slowly processed. This limitation could be overcome for *E. coli* $\beta$-lactamase by overexpression of SipS (van Dijl et al., 1992; Bolhuis et al., 1999a). This overexpression could solve one of the bottlenecks of hTRAIL production, because part of hTRAIL that is found in the medium fraction still has its signal sequence attached. Optimising the post-translocational folding of mature hTRAIL into a stable and active conformation might be achieved by overexpression of the extracellular lipoprotein PrsA as unproductive interactions with the wall are assumed to be prevented by this protein (Wahlström et al., 2003). An increase in PrsA production has been shown to decrease the degradation of some exported proteins (like AmyQ, single chain antibodies, pneumolysin, and *Bacillus anthracis* recombinant protective antigen) resulting in increased yields of these proteins in the culture medium (Wu et al., 1998; Williams et al., 2003; Sarvas et al., 2004; Vitikainen et al., 2005). It has to be noted however that not all *B. subtilis* secretory proteins are PrsA dependent (Vitikainen et al., 2004; Harwood and Cranenburgh, 2008). As PrsA has sequence similarity to prokaryotic and eukaryotic peptidyl-prolyl cis-trans isomerases (PPIases) of the parvulin family (Wahlström et al., 2003; Tossavainen et al., 2006) it may be assumed that the chaperone increases the folding of proteins with cis-prolyl residues (Harwood and Cranenburgh, 2008). However, Sarvas et al. (2004) conclude from different studies that the PPIase activity is of minor importance for the function of PrsA in vivo. The need for PrsA for efficient folding of proteins seems to be associated with some feature of the micro-environment at the membrane-wall interface (Wahlström et al., 2003; Sarvas et al., 2004; Vitikainen et al., 2004).

One major problem for obtaining high extracellular levels of a desired protein is the presence of high levels of extracellular proteins. Eight extracellular proteases have been identified in *B. subtilis* to date, namely: AprE, Bpr, Epr, Mpr, NprB, NprE, Vpr, and WprA. Recently, the degradation of a heterologous protein (the PreS2 antigen of human hepatitis B virus) by extracellular protease activity of AprX during the late stationary growth phase was demonstrated. It was proposed that AprX leaks to the culture medium probably upon cell lysis (Kodama et al., 2007). These studies show that still additional proteolytic activity
can interfere with protein production from the *B. subtilis* cell factory even in multiple protease negative strains such as WB800.

Paradoxically, the most important protein quality control system of *B. subtilis* is also the major obstacle to effective production and secretion of many heterologous proteins. This control system is formed by the secretion stress response and wall-associated and extracellular proteases that lead to the refolding or degradation of extracytoplasmic misfolded proteins. The main proteases involved in the degradation of misfolded proteins are WprA, HtrA and HtrB. The first is thought to be induced in response to secretion stress (Harwood and Cranenburgh, 2008) and the last two are regulated by the CssRS two-component system. HtrA has been proven to have a chaperone-like activity besides its proteolytic activity, this protein might assist misfolded proteins to recover their correct configuration (Antelmann *et al.*, 2003). The modulation of this quality control system creates possibilities for the optimisation of (heterologous) protein production. However, it has to be kept in mind that the regulation of this system seems quite delicate and that high-level production of a correctly folded and, most importantly, active protein will always require quality control steps.

In conclusion, the studies described in this thesis provide novel insights into the optimisation of *B. subtilis* as a cell factory. In the past decennia, much of the work on *B. subtilis* focussed on enhancing the exploitation potential of this Cell Factory for biotechnological applications. Mechanisms of protein synthesis, targeting, translocation, release, folding, and degradation were studied and revealed. The obtained data on protein production and secretion point out clearly that for each individual recombinant protein different factors limit the production levels. Therefore, the engineering of *B. subtilis* cell factories to some extent requires a protein-specific approach. Educated guesses can be made to improve the production or secretion of a certain protein, but the outcome of such an attempt is not fully predictable. Furthermore, the opportunities to optimise a *B. subtilis* cell will not be indefinite with respect to the fact there will be cell viability-associated limitations to the regulation of the different mechanisms involved in protein translocation and protein quality control.

As stated above a lot of knowledge has been gained during the last decades, however, more knowledge still has to be gained about folding of the proteins (either inside the cell or at the membrane-cell wall interface), the influence of the cell wall environment (Hyryäläinen *et al.*, 2007), and release of the mature protein into the growth medium. Also, it is still not clear whether the secretion stress response is “a good or a bad thing” for obtaining high-level and high-quality heterologous protein yields. Furthermore, to ensure proper folding
and forming of disulphide bridges, the quest in *B. subtilis* for the isomerase activity that was shown for EGF production in *Bacillus brevis* (Miyauchi et al., 1999) is also still ongoing.

With the current knowledge available, the road is paved to further optimise *B. subtilis* as a Cell Factory. It has become possible to rationally choose and design an optimal production system and it has become clear that the properties of each desired protein have to be taken into account. The final demonstration of the suitability of the BACELL factory for producing therapeutic proteins awaits the first FDA approved pharmaceutical protein produced in *Bacillus* brought to the market (Chapter 2, Westers et al., 2004b).