Chapter 8

General summary and discussion
General summary and discussion

For bacteria to survive in their natural habitat it is essential to transport a variety of proteins, including enzymes, from the cytoplasm to the cell envelope and the extracellular environment. These exported proteins facilitate the uptake of nutrients and serve to maintain structural features of the cell (Richardson et al. 2015; Yuan et al. 2010). The phenomenon of protein export is used in a wide range of commercial, pharmaceutical and biotechnological applications (e.g. the textile, brewing and food industries), where biologically active enzymes are overproduced, secreted and subsequently purified for specific applications. To increase the production yield, the protein secretion process in bacterial cell factories can be optimized by genetic engineering. Together with improved production yields, optimization of the protein secretion pathway may provide the possibility to create adapted or modified enzymes and to generate a more sustainable production pipeline. One bacterium used intensely in biotechnological processes is the Gram-positive bacterium Bacillus subtilis. This ‘cell factory’ is capable of secreting bulk amounts of protein (over 25 g/l) into the fermentation broth (Van Dijl and Hecker 2013), which greatly simplifies their downstream processing. Due to the well-developed secretion system of B. subtilis, this bacterium has become popular for the bulk production of proteins, as exemplified by proteases that are applied in detergents, amylases used in the food industry, or xylanases with applications in paper production (Van Dijl and Hecker 2013; Harwood and Cranenburgh 2007).

Although B. subtilis is very capable of secreting high amounts of protein into the extracellular milieu, the fact that it produces an extensive number of proteolytic enzymes that are cell envelope-associated or secreted is also a disadvantage, since this may lead to product degradation (Harwood and Cranenburgh 2007; Pohl et al. 2013). To overcome product degradation, B. subtilis strains have been engineered that lack most of these proteases (Westers et al. 2006; Pohl et al. 2013). Unfortunately, it appears that such protease-deficient strains show increased sensitivity to cell lysis, which is probably the main reason why they are not intensely used as industrial cell factories (Krishnappa et al. 2013).

Although B. subtilis is successfully applied in the production of bulk amounts of functional enzymes from closely related species, the production of more delicate heterologous proteins (e.g. antigens for immuno-therapies) can be difficult due to proteolytic activity. This opens a niche for another bacterial cell factory, namely Lactococcus lactis, a food-grade bacterium that is mainly used in the in the production of cheese, dairy products and probiotics. Since L. lactis only possesses two extracellular proteases, which can also be eliminated from the cell, the proteins produced by L. lactis are apparently less subject to proteolysis (Steen et al. 2005). However, the use of L. lactis as a cell factory in the biotechnological industry setting is currently restricted by low production yields (mg/l range) (Neef et al. 2014). Considering the potential benefits, this calls for optimization of the protein secretion process.
in *L. lactis*. For example, this thesis reports on the beneficial effects of removing the autolysin AcmA and the extracellular protease HtrA on strain stability and extracellular protein production. Also, a suite of effective expression vectors was developed for the production of extracellular proteins, allowing the construction of stable fusion proteins with different exchangeable tags for protein detection and purification.

The PhD research described in this thesis was aimed at the optimization of protein production by *L. lactis* and *B. subtilis*. To be able to engineer strains with enhanced capacity for protein production it is important to obtain as much knowledge as possible about the respective production platforms as they are used today. This was the main objective for the literature and bioinformatics analyses described in Chapter 1, which reports on an in-depth comparative analysis of the protein secretion pathways and the secretory proteins of *B. subtilis* and *L. lactis*. Much emphasis was placed on the properties of signal peptides that direct protein export and the signals that may retain proteins at particular subcellular locations, taking advantage of the previously published genome data. Different properties of the signal peptides and their mature cargo were compared, from which it was concluded that secreted proteins of *L. lactis* contain significantly longer signal peptides than secreted proteins of *B. subtilis*. Of note, for secretory protein production in *B. subtilis*, a wide variety of different signal peptides has been applied in industry, whereas for secretory protein production in *L. lactis* the preferred signal peptide is obtained from the major secreted protein Usp45.

To explore the possibilities for protein production in *L. lactis*, the strain PA1001 was applied in combination with a suite of improved expression vectors (Chapter 2, 3 and 4). The optimized *L. lactis* strain P1001 lacks genes encoding the major HtrA protease and the autolysin AcmA, and it was successfully used for production of twelve different surface-exposed and secreted antigens from *Staphylococcus aureus* (Chapter 2 and 3). These antigens were selected as potential targets for vaccination studies and/or to monitor adaptive human immune responses against *S. aureus*. In particular, Chapter 2 describes both intra- and extracellular production of proteins in *L. lactis* PA1001 in comparison to the more widely used NZ9000 strain. In particular, a cell surface-binding tag consisting of three LysM cell wall-binding domains, an AVI-tag for site-directed biotin labeling, and a hexahistidine-tag (His6) for purification of the produced protein by metal affinity chromatography were fused to the tested *S. aureus* antigens. Using the secretion signal of Usp45, it was possible to direct the proteins to the translocation machinery, resulting in efficient secretion and subsequent purification from the growth medium. The pipeline for extracellular protein production was further optimized by introducing second- and third-generation vectors as described in Chapters 3 and 4. The latter vectors include an additional Strep-II tag for high-affinity binding of secreted proteins using the Strep-Tactin technology (Schmidt and Skerra 2007) and an AVI-tag at flexible positions for protein labeling. The combined vectors enable the production of proteins with N- or C-terminal His6-tags, where the C-
terminal His$_6$-tag can be removed using the tobacco etch virus (TEV) protease. Notably, it turned out that the nature and the relative position of tags can have a major impact on the production-rates and stability of heterologously expressed proteins in *L. lactis*.

An interesting observation was that *L. lactis* allowed production of the naturally phosphorylated IsdB protein of *S. aureus*. The IsdB secreted from *L. lactis* was also phosphorylated, although at a different position (Tyr311 instead of Tyr440 or Tyr444 (Basell et al. 2014)). The reason for this difference is presently not clear, but it could be due to differences in the preparation of protein samples used for mass spectrometry analyses, since Basell et al. used a gel-free proteomics approach, whereas the IsdB produced in *L. lactis* was extracted from an LDS-PAGE gel. Another possible reason for the differential phosphorylation could be that the IsdB protein produced in *L. lactis* was provided with the Usp45 signal peptide and contained a TEV protease cleavable His$_6$-tag. Lastly, it is also conceivable that the kinase responsible for the phosphorylation of IsdB in *S. aureus* may have a specificity that differs from the specificity of the respective kinase in *L. lactis*. Although there are some factors that might influence the site of phosphorylation, this study described for the first time that *L. lactis* is capable of phosphorylating a heterologous exported protein.

To illustrate the application potential of the Strep-II tag implemented in the vector set presented in Chapter 4, the *S. aureus* LysM protein provided with this tag was successfully used in enzyme-linked immunosorbent assays (ELISAs) with human sera. This concept was expanded in the studies described in Chapter 5, where ten non-covalently cell wall-bound His$_6$-tagged *S. aureus* proteins were extracellularly produced in *L. lactis* and, subsequently, used to profile human antibody responses. Of note, the heterologous production of these proteins in *L. lactis* did not interfere with their ability to bind the *S. aureus* cell wall as was shown by their incubation with *S. aureus* cells. Subsequently, the purified proteins were used to profile antibody levels in plasma samples of patients suffering from the genetic blistering disease Epidermolysis bullosa (EB) and healthy volunteers. The plasma from EB patients showed significantly higher antibody levels against the non-covalently bound proteins of *S. aureus* than those of the healthy volunteers, suggesting that the EB patients were more exposed to these antigens. This is consistent with the fact that the respective EB patients are heavily exposed to *S. aureus* due to chronic colonization by oftentimes multiple types of *S. aureus* (van der Kooi-Pol et al. 2012; van der Kooi-Pol et al. 2013; van der Kooi-Pol et al. 2014).

While Chapters 2 to 4 of this thesis mainly focus on the optimization of protein production by improving host-vector systems in *L. lactis*, the following Chapters 6 and 7 address possible improvements of the general secretion (Sec) pathway of *B. subtilis*.

Chapter 6 focuses attention on the intramembrane protease RasP as a major secretion bottleneck in *B. subtilis*. In particular, RasP was shown to set limits to the efficient extracellular production of two proteins, namely the protease ‘Properase’ and an AmyAc type amylase. The production efficiency of
these proteins in the wild-type background was low, which is at least partly due to reduced cell growth and/or viability at late stages of the fermentation process. When \text{RasP} was overexpressed in the respective production strains, these negative effects were overcome. As a result, the secretion of the \text{AmyAc} type amylase was enhanced up to 10-fold. These findings represent the first evidence that overexpression of a protease, which is active within the plane of the cytoplasmic membrane of a bacterial cell factory can improve protein production, likely by cleaning the cytoplasmic membrane from signal peptide remnants. Another reason for this finding could be the possible intramembrane degradation of malformed or mis-located secretory precursors or membrane proteins, which may interfere with essential membrane processes (Wadenpohl and Bramkamp 2010). Lastly, since \text{RasP} is involved in the induction of $\sigma^\vee$ and $\sigma^\mu$-dependent genes, potentially altered expression of these genes upon \text{RasP} overexpression may contribute to the improved production of \text{Properase} and \text{AmyAc} (Zweers et al. 2012; Hastie et al. 2013; Heinrich et al. 2009). Although it remains to be shown how exactly \text{RasP} plays a role in protein translocation, it is clear from the results presented in Chapter 6 that overproduction of \text{RasP} can be exploited to boost protein production and secretion in \text{Bacillus}. This might also be true for other intra-membrane proteases, which would open up a new chapter in the ‘tale of cell factory engineering’.

Lastly, in the studies for Chapter 7, the possible roles of non-essential Sec pathway components and cell envelope-associated proteases in high-level enzyme secretion in \text{B. subtilis} were investigated. To this end, the respective genes were deleted and the secretion potential of the resulting mutant strains was investigated. From the results, it was concluded that, in addition to \text{RasP}, also the translocase components \text{SecDF} and \text{SecG} are critical for high-level production of secreted enzymes like \text{AmyE}, \text{AmyL} and \text{BPN’}. Since \text{SecDF} and \text{SecG} are part of the translocation channel, the effects of their deletion are likely to relate to defects in the translocation process. In the case of \text{BPN’}, also the general chaperone \text{DnaK} was found to be important for optimal secretion, but here it is presently not clear whether this represents a direct or an indirect effect of the \text{DnaK}-deficiency. For the first time, the effects of secretion machinery mutations or the absence of various cell envelope-associated proteases on the \text{CssRS}-dependent protein secretion stress response were measured, making use of the \text{HtrA} and \text{HtrB} proteins as markers for this type of stress. Unexpectedly, mutation of \text{sipV} had the strongest effect on the cellular levels of \text{HtrA} and \text{HtrB}, especially in the non-producing strains. The main advantage of the applied Western blotting approach is that the immunodetection with antibodies against \text{HtrA} and \text{HtrB} directly monitors the cellular levels of these quality control proteases. However, the disadvantage is that \text{HtrA} and \text{HtrB} need to be exported themselves, making them sensitive to differential translocation efficiencies in mutant strains with secretion machinery defects. While this complicates the interpretation of the obtained results to some extent, assessment of the \text{HtrA} and \text{HtrB} protein levels does show that defects in the secretion machinery do not elicit a massive \text{CssRS-}
dependent secretion stress response. To what extent other stress responses are elicited by the investigated mutations remains to be investigated.

In conclusion, the results described in this PhD thesis provide new clues for optimizing protein production in L. lactis and B. subtilis. First of all, the use of an L. lactis strain that is reduced in autolysis and extracellular proteolysis, together with a versatile vector suite simplifies the production of tagged proteins that are vulnerable to degradation. This opens up possibilities for the exploitation of L. lactis in the production of antigens for vaccination against important pathogens, such as S. aureus. Also, the knowledge gained from the previous and present studies on the roles of non-essential secretion machinery components in B. subtilis for extracellular protein production can be translated for enhanced protein production in L. lactis. In this respect, it is helpful that the protein secretion machinery of L. lactis and B. subtilis is similar but not identical, allowing for the exchange of particular components. The feasibility of such an approach is underscored by a previous study showing that the expression of B. subtilis SecDF in L. lactis can indeed lead to improved protein production (Nouaillé et al. 2006). Conversely, the investigation on B. subtilis RasP shows that there are still secretion pathway components that can be engineered to improve protein production, focusing attention on membrane proteases. Indeed, a recent study in B. licheniformis showed that the overproduction of the signal peptide peptidase SppA resulted in improved protein production (Cai et al. 2017).

Altogether, it seems that there is an open ending to the here presented ‘tale of two cell factories’. Firstly, it appears worthwhile to further explore the improved secretion properties of the so-called mini-Bacillus strain PG10 (Aguilar Suarez et al. 2018). Since this mini-factory lacks about 36% of the Bacillus genome, it represents an attractive platform to test possible applications of key secretion pathway components, like SecDF, SecG or RasP. Likewise, the recently explored non-classical secretion pathway(s) could open new doors to enhanced secretory protein production, not only in B. subtilis (Chen et al. 2016; Yang et al. 2011; Zhao et al. 2017), but also in L. lactis. Importantly, such future studies should not remain limited to the secretion machinery hardware, but they may also take into account the management of cellular resources. As shown in a recent study by Bulovic et al., the latter can be facilitated by whole-cell bacterial resource allocation models (Bulovic et al. 2019).

References
Chapter 8


Van Dijl JM, Hecker M (2013) Bacillus subtilis: from soil bacterium to super secreting cell factory. Microbial Cell Factories 12: 3


