Chapter 10

Summary and general discussion

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Pathways for protein secretion in *B. subtilis*

In the last decade, *B. subtilis* has been broadly deployed for secretome prediction and analysis studies (Antelmann *et al.*, 2000; Antelmann *et al.*, 2001; Antelmann *et al.*, 2003; Hirose *et al.*, 2000; Tjalsma *et al.*, 2000; Tjalsma *et al.*, 2004; van Wely *et al.*, 2001). This work has resulted in the establishment of several different pathways for protein secretion of which the Sec pathway was clearly the most preferred route. Despite these findings, not all secreted proteins that are found in the growth medium of *B. subtilis* can be explained by the existence of these pathways alone. This suggests that there are still unknown pathways that contribute to protein secretion in *B. subtilis*. Furthermore, these previous proteomic studies were performed under laboratory conditions with limited variation, which does very little to mimic the changing environments that *B. subtilis* encounters in the soil. It is therefore likely that the actual secretome of *B. subtilis* is far from fully understood. A few hints to the truth of that statement are encountered in this thesis.

In chapter 7, the large conductance mechanosensitive channel protein MscL was investigated for its possible contribution to protein secretion. The predicted pore diameter of this channel of about 30 Å is potentially large enough to allow the passage of small proteins. In fact, it has been suggested that the MscL channel of *E. coli* is able to secrete small proteins such as the thioredoxin protein TrxA (Ajouz *et al.*, 1998; Berrier *et al.*, 2000; Lunn and Pigiet, 1982). Based on the size of the crystallized *B. subtilis* TrxA-dimer that is described in chapter 6, the size of the monomeric *B. subtilis* TrxA would be approximately 30x30x30 Å, indicating that the MscL pore might just be large enough for the passage of this protein and other proteins with similar or smaller sizes. However, no direct evidence for protein export via the MscL channel could be obtained through proteomics. Conversely however, the results in chapter 9 indicate that MscL may serve as the cellular entry gate for the lantibiotic sublancin 168, which would then travel through this pore from the outside to the inside of the cell. Notably, the exact contribution of the MscL channel to the secretion of proteins in *B. subtilis* is very difficult to assess due to the difficulty in distinguishing secreted proteins from proteins released by cell lysis. While there is no conclusive evidence for protein export via MscL, there was also no indication found that MscL would not be able to secrete proteins. Intriguingly, the analysis of an mscL mutant strain in chapter 7 revealed the existence of a new, as yet unidentified, pathway for selective protein release.

The determination of protein release via this unidentified channel was carried out under osmotic shock conditions. This may not be the situation that is encountered by cells during standard laboratory culturing, but it is for sure a situation that is frequently encountered in the ecological nice that *B. subtilis* inhabits – the soil - for example during rain. It might even also be encountered by *B. subtilis* during industrial fermentations in large fermentors were fluctuations in pressure and osmosis are hard to prevent. These studies therefore show that the actual secretome of *B. subtilis* is likely to be far more
complex than so far encountered under standard laboratory growth conditions. Future secretome research on establishing the secretome of *B. subtilis* should therefore also focus on these alternative, ecologically and environmentally relevant growth conditions of *B. subtilis* that differ substantially from the standard culture conditions in the laboratory.

Another, often overlooked view in studies on secretome biogenesis is the possible interdependency of established secretion pathways. The ability of proteins to use multiple pathways for secretion has not been truly explored before and there are no reported cases of proteins that are able to travel via multiple routes. The results obtained using the hyper-secreted LipA protein in chapter 3 are thus unprecedented. The LipA hyper-secreting strain was serendipitously obtained during the studies described in chapter 2 and was shown to secrete LipA via two distinct routes: the general Sec pathway and the Tat pathway. Although this mutant produced LipA at much higher levels than the wild-type strain, it does illustrate that the same type of protein can be secreted via different pathways. This might then also be the case for other proteins that are secreted at elevated levels under particular, as yet unidentified, conditions. Most likely, this would require the presence of appropriate recognition signals for both secretion pathways as seems to be the case for Lip, which has a signal peptide that can be accepted by both the Sec and the Tat pathway. It was previously established experimentally that the Sec pathway transports most of the secreted proteins of *B. subtilis* (Antelmann et al., 2001; Tjalsma et al., 2000; Tjalsma et al., 2004). This was mostly determined on the basis of dependency on SecA for processing of these secreted proteins. SecA dependency has been shown for many potential Tat substrates, including LipA, and these were then disregarded as possible Tat substrates. Although this might still be correct, the results obtained for the hyper-produced LipA in chapter 3 call for a careful reinvestigation of the Tat-independent secretion of the 69 *B. subtilis* proteins with potential RR-motifs in their signal peptides.

Taken together, the results presented in this thesis focus on the possibility that secretion pathway choice can be determined by environmental (e.g. osmotic shock) and intracellular conditions (e.g. hyper-production). This provides new views on the established protein secretion pathways and urges the search for new routes that contribute to secretome biogenesis in *B. subtilis*. This search for new secretion routes and the identification of the environmental or cellular conditions that influence the usage of particular secretory pathways will be a challenge for future studies. An important challenge will be the establishment of a more complete picture of the secretome of *B. subtilis* that will not only provide insights on how this organism copes with the ever-changing environments of its natural habitat, but will also provide valuable leads for more controllable secretion systems within industrial protein production settings.
Disulfide bond handling in B. subtilis

Numerous secreted proteins found in nature are dependent on the formation of disulfide bonds for their stability and function (Collet and Bardwell, 2002; Ritz and Beckwith, 2001). Especially eukaryotic organisms secrete many proteins that contain such bonds. B. subtilis was found to secrete only a few proteins that contain disulfide bonds (i.e. ComGC and sublancin 168) (Dorenbos et al., 2002; Meima et al., 2002). However, for its purpose as production host of heterologous proteins, the study of disulfide bond formation in B. subtilis is very relevant. For a long time it was believed that the formation of disulfide bonds occurred spontaneously due to exposure to oxidation. This paradigm changed however with the discovery of specially evolved enzymes known as thiol-disulfide oxidoreductases (TDORs) that facilitate their formation and breakage. The current view is that each of these TDORs are unique enzymes that function either as oxidases, reductases or isomerases in fixed, separate pathways. The combined work presented in this thesis shows that this view is too simplistic and needs expansion (reviewed in chapter 1). In addition, several findings are reported that give a more profound understanding of disulfide bond formation in B. subtilis.

The oxidation pathway in Gram-negative bacteria is mainly based on studies in E. coli and is thought to be represented by a central periplasmic thiol-disulfide oxidoreductase (DsbA) which is kept oxidized by a fixed partner quinone oxidoreductase (DsbB) located in the cytoplasmic membrane. In Gram-positive bacteria, mainly based on research on B. subtilis, this pair-wise role was thought to be played by the thiol-disulfide oxidoreductase BdbD and the quinone oxidoreductase BdbC, both located in the membrane. These TDORs were both shown to be required for the oxidation of ComGC and E. coli PhoA (Bolhuis et al., 1999; Meima et al., 2002). However, the data presented in this thesis show that the actual situation is not as straightforward as previously believed. When the TDOR systems of all the low-GC Gram-positive bacteria were compared in chapter 2, it became clear that they had evolved differently. Some members of this phylum – the Firmicutes - such as B. subtilis and Bacillus cereus, indeed contain BdbD-BdbC pairs, while others contain only a BdbC homologue (e.g. Bacillus clausii and Bacillus halodurans) or only a BdbD homologue (e.g. Staphylococcus aureus and Staphylococcus epidermidis). Interestingly, when the S. aureus BdbD homologue (DsbA) was functionally expressed in B. subtilis it was found to be able to complement for both BdbD and BdbC in at least three Bdb-dependent processes (chapter 2). Thus, the S. aureus BdbD homologue did apparently not need a BdbC homologue for reoxidation. Importantly, studies with cells grown in minimal medium (chapter 2) revealed that S. aureus DsbA can be reoxidized by active redox compounds in the medium instead of redox-active partner proteins. This indicated that TDORs in bacteria have evolved in various ways and do not conform to one single format. In fact, it became evident that the “standard” B. subtilis laboratory strain (168) is actually an exceptional case in the sense that it uniquely carries the genes for two additional oxidative TDORs, BdbA and BdbB, which are not encountered in other bacteria (chapter
2). BdbB is homologous to BdbC and BdbA has a topology that resembles the topology of BdbD. Since the bdbA and bdbB genes are located on the SPβ prophage, it was assumed that they are exclusively required for the formation of the disulfide bonds in the lantibiotic sublancin 168, which is also encoded by the SPβ prophage (Paik et al., 1998; Stein, 2005) (chapter 8&9). The studies in chapter 2 together with previous work (Dorenbos et al., 2002) show that this is not exactly the case. Although BdbB is of primary importance for the secretion of active sublancin 168, its parologue BdbC was also found to contribute to active sublancin 168 secretion (Dorenbos et al., 2002) (chapter 2). Furthermore, BdbA was found to be dispensable for sublancin 168 production and, in fact, the biological function of BdbA is as yet unknown. Although BdbA resembles BdbD with respect to membrane topology and the presence of a typical TDOR active site, it is questionable whether BdbA does function as an oxidative TDOR. Importantly, the remarkable finding that the presence of BdbB is sufficient for the production of active sublancin 168 revealed that even in B. subtilis, which does contain both the BdbD and BdbC proteins, the combined use of these two TDORs is not always required. Together, this led to the proposition that TDORs behave as functional modules that can act both independently and in a concerted manner (chapter 2). Their activity is determined by their molecular context and the combined presence of other TDORs (chapter 1). Recent observations by the groups of Bardwell and Collet indicate that this paradigm is likely to apply also for E. coli (Vertommen et al., 2008). It was shown that, next to the central oxidase DsbA, the disulfide isomerase DsbC can also participate in protein oxidation. The substrate specificities of these TDORs, like those of Gram-positive bacteria, are not fixed either but also seem to depend on the presence of other TDORs. Functional TDOR modules as encountered in B. subtilis and other Low-GC Gram-positive bacteria thus seem to represent a common theme in disulfide bond formation and illustrate the complexity in disulfide bond formation that can also be expected in other organisms.

In contrast to E. coli, B. subtilis has not been shown to contain a protein disulfide isomerase pathway. At least it does not contain direct homologues of the E. coli isomerases. However, B. subtilis does contain reductive TDORs in the cytoplasmic membrane, which have been shown to be involved in reducing apocytochrome c and spore cortex proteins (Moller and Hederstedt, 2006). Interestingly, the central TDOR in this system, CcdA, together with an associated TDOR (ResA or StoA) can be seen as a functional and structural homologue of the E. coli DsbD TDOR, which transfers electrons to the disulfide isomerase DsbC (Katzen et al., 2002). Previously, the homologue of another such CcdA-associated TDOR, YneN, was shown to perform protein disulfide oxidoreductase-like activity in Brevibacillus choshinensis (B. brevis). Incubation of non-native human epidermal growth factor (hEGF) containing incorrect disulfide bonds with B. choshinensis cells over-expressing YneN, or co-expression of the two proteins, increased the final yield of active hEGF protein (Miyauchi et al., 1999; Tanaka et al., 2003). However, co-expression or deletion of the B. subtilis YneN together with the expression of E. coli PhoA by B. subtilis did not have any detectable effect on the yield of active PhoA.
(Dorenbos et al., unpublished observations, chapter 5). It remains therefore an intriguing issue whether *B. subtilis* contains a system for protein disulfide isomerase activity or not.

Irrespectively of whether the CcdA-associated TDORs have protein disulfide isomerase activity or not, it is possible that these TDORs have an (indirect) influence on oxidative protein folding since there is crosstalk between the reductive and oxidative TDORs in the membrane. Hederstedt and co-workers have established that mutations in *bdbC* or *bdbD* suppress mutations in *ccdA* and *resA*, which affect the reduction of apocytochrome *c* (Erlendsson and Hederstedt, 2002). Whether this crosstalk takes place only indirectly via shared substrates such as apocytochrome *c*, or also directly, would be an interesting topic for future study. The effects of mutations in CcdA or ResA on the oxidative folding of PhoA have for example not yet been tested. Further evidence for TDOR cross talk was generated in chapter 5 where it was observed that depletion of the cytoplasmic reductase TrxA influenced the redox state of BdbC. This influence of electron transport from TrxA is likely to be directed via the reductive membrane TDORs as was shown for *E. coli* DsbD (Katzen and Beckwith, 2000; Ito and Inaba, 2008). Direct electron transport from TrxA to BdbC can, however, not be excluded eventhough interactions between oxidative TDORs and thioredoxins have not (yet) been reported in literature.

To study interactions between thioredoxin and its substrates, we have established the mixed disulfide fishing technique for *B. subtilis* in chapter 6. Thioredoxin variants with single or double cysteine-to-serine mutations were purified and shown to be able to trap stable interactions between thioredoxin and possible substrates. This technique can now be used in future studies to generate a more complete picture of the TDOR interaction networks in *B. subtilis*.

**Secretion of heterologous proteins by B. subtilis**

The natural capacity of *B. subtilis* to secrete many industrially relevant enzymes in high amounts into its growth medium, and the relative ease by which it is cultured and genetically manipulated, have made *B. subtilis* one of the preferred hosts for industrial protein secretion (Meima et al., 2004; Schallmey et al., 2004; Zeigler and Perkins, 2008). While several strategies have been developed to allow the efficient production of relevant proteins by *B. subtilis*, serious bottlenecks do exist, especially when heterologous proteins are produced by *B. subtilis*. These bottlenecks include the efficient expression of the heterologous gene, the translocation of the foreign proteins across the plasma membrane, the release of the exported proteins from the cell surface into the surrounding medium and the correct formation of disulfide bonds (Meens et al., 1997; Puohiniemi et al., 1992; Saunders et al., 1987; Sarvas et al., 2004). In this thesis several strategies are presented that may help in relieving these bottlenecks.

When it was attempted in chapter 2 to express the *S. aureus* DsbA lipoprotein in *B. subtilis* using the native *dsbA* ribosomal binding site (RBS) and signal sequence, no
detectable DsbA production was observed. This might either be related to inefficient recognition of the *dsbA* RBS by the translation machinery of *B. subtilis*, or to a poor recognition of the lipoprotein signal peptide of DsbA by the secretion machinery of *B. subtilis*. In contrast, when the ribosomal binding site and signal sequence were replaced with those encoding for the *B. subtilis* lipoprotein MntA, good DsbA production in *B. subtilis* was achieved. The use of expression and targeting sequences of native proteins of *B. subtilis* to direct the production of heterologous proteins in this bacterium seems therefore a good strategy, and the present approach seems very well suited for the production of lipoproteins. Alternatively, this approach may be applicable for the introduction of relevant heterologous protein folding catalysts at the membrane-cell wall interface.

Importantly, certain heterologous signal peptides are efficiently recognized in *B. subtilis*, and these can even be used to direct the translocation of particular heterologous proteins, as was shown in chapter 4. In this chapter, the signal sequence of a *Staphylococcus hyicus* lipase was shown to direct the efficient translocation of *E. coli* PhoA produced in *B. subtilis* substantially more efficiently than the authentic PhoA secretion signal.

Interestingly, in chapter 3 we serendipitously obtained a *B. subtilis* mutant that secreted the native extracellular esterase LipA in over a 100-fold increased amounts compared to wild-type levels, without any signs of inefficient translocation. This suggests that the LipA secretion signals can potentially also be used for the efficient translocation of other proteins. Furthermore, it is tempting to speculate that the LipA hyper-producing strain itself could also be used as a host for the secretion of other proteins. It would therefore be very interesting for future studies to characterize the mutation(s) that lead(s) to the increased LipA production, and to try to adopt this strain for the development of a new expression system for protein production.

An additional strategy for more efficient secretion of heterologous proteins by *B. subtilis* is presented in chapter 4 where the secretion of *E. coli* PhoA fused to the pro-peptide of the *S. hyicus* lipase was tested. Pro-peptides are generally known to serve in the post-translocational folding process to achieve an active and stable form of the secretory protein (Sarvas *et al.*, 2004; van Dijl *et al.*, 2001). However, it was observed in chapter 4 that the presence of the pro-peptide also resulted in significantly improved export of PhoA from the cytoplasm, while a control PhoA protein expressed without this pro-peptide remained to a larger extent membrane attached. This is consistent with the previously reported observation that pro*sup* supports the efficient secretion of *E. coli* OmpA both by *B. subtilis* and *Staphylococcus carnosus* (Meens *et al.*, 1993; Meens *et al.*, 1997). Taken together, the use of dedicated pro-peptides in combination with pre-peptides, such as the ones mentioned above, seems therefore an efficient strategy for heterologous protein secretion by *B. subtilis*.
Summary and general discussion

Secretion of disulfide bond-containing proteins by *B. subtilis*.

The above discussed strategies for increased protein targeting and translocation across the membrane were shown to contribute greatly to increased heterologous protein secretion in *B. subtilis*. There is one process that occurs after translocation that will however still comprise a bottleneck in the production of many heterologous proteins. This process is the correct formation of disulfide bonds. *B. subtilis* is inefficient in forming disulfide bonds, and since many biopharmaceutical proteins depend on these bonds for their activity and stability, this obstacle puts a serious limitation to the use of *B. subtilis* for heterologous protein production. A large emphasis in this thesis was therefore put on this issue.

The formation (oxidation) of disulfide bonds is catalyzed by TDORs. Although this enzymatic activity is much faster and more specific than the spontaneous oxidation by oxygen, there still seems to be a threshold to the level of TDOR activity. Accordingly, when heterologous proteins containing disulfide bonds are produced in *B. subtilis* in high amounts, it is likely that this system gets overloaded, resulting in incorrect disulfide oxidation and misfolding of the proteins. These incorrectly folded proteins are then quickly degraded in the highly proteolytic extracellular environment of *B. subtilis*. This is illustrated in chapter 4 by studies on the disulfide bond containing prepro\textsuperscript{lip}-PhoA when secreted by *B. subtilis*. Although the PhoA protein is efficiently translocated across the membrane, the many degradation products indicated that a large fraction of the secreted proteins was apparently not correctly folded.

The most straightforward solution to solve this bottleneck would appear to artificially increase the number of copies of the oxidative TDORs in the cytoplasmic membrane. Previous secretion studies in *B. subtilis* showed, however, that the increased expression of either one of the four *bdb* genes did not result in better protein folding of *E. coli* PhoA (Darmon et al., 2006; Dorenbos et al., 2002; Meima et al., 2002). Even the combined expression of different Bdb proteins was shown to have no beneficial effects on the levels of active PhoA obtained (chapter 5 and unpublished observations).

In chapter 2 we discovered that not all organisms contain the same TDOR pathways. An alternative was therefore presented by introducing TDORs in *B. subtilis* from a pathway that did not depend on the same quinone oxidoreductases as the Bdb proteins. And indeed, introduction of the DsbA protein from either *S. aureus* or *S. carnosus* in *B. subtilis* resulted in higher levels of active extracellular PhoA (chapter 5). The addition of cysteine (which can serve to reoxidate DsbA as described in chapter 2) to the medium was shown to increase the extracellular PhoA levels even further. This showed that it is possible to combine different pathways for disulfide bond formation in order to increase the oxidative potential of the cell.

Finally, we reasoned that instead of increasing the level of oxidative TDORs, it might also be possible to decrease that of the reductive ones, in order to achieve higher levels of disulfide bond formation. This was indeed shown to be the case when the
cytoplasmic reductase TrxA was depleted, but not when we depleted any other cytoplasmic or membrane reductases (chapter 5). Depletion of TrxA was shown to result in a higher level of BdbD oxidation, suggesting that this might be due to less electron transport from TrxA to BdbC/BdbD (chapter 5). It should be noted that we did not test the depletion of CcdA and ResA since these proteins were shown to be involved in reducing cytochrome c (Erlandsson et al., 2003; Schiott et al., 1997). In retrospect, it is interesting to also test the depletion of these two TDORs to investigate whether the depletion effect of TrxA on BdbD/BdbC is directed via the CcdA/ResA pathway or directly at the level of the BdbC/D proteins.

The studies in this thesis were focused on optimized production of oxidative TDORs and depletion of the reductive ones. There is however likely to be a limit to how far such alterations can be imposed upon cells before the level of this oxidation becomes toxic. For example, when we depleted the cells of TrxB, the TrxA reductase, severe growth defects were observed (unpublished results). Also, TrxA is essential for growth and viability under most conditions (Moller and Hederstedt, 2008; Mostertz et al., 2008). Further increase in the level of disulfide bond formation by B. subtilis should therefore be obtained via alternative strategies. In this light, it is interesting to note that in B. brevis, the overproduction of members of the reductive TDOR pathway (YneN, CcdA and even TrxA) resulted in improved production levels of active hEGF, which contains three disulphide bonds (Miyachi et al., 1999; Tanaka et al., 2003). This is supposedly due to the isomerase activity of these proteins. This method however required the incubation of B. brevis cells for two to three days with the medium containing the hEGF. Such an approach does not seem feasible for B. subtilis. Even if the homologous pathway of these proteins in B. subtilis would have protein disulfide isomerase activity, the incorrectly folded proteins would be degraded very rapidly. Future studies to asses the contribution of the reductive TDOR pathway on protein isomerization could however potentially be carried out using a total protease knockout strain such as the WB800 strain (Murashima et al., 2002), or in the presence of protease inhibitors (Westers et al., 2008). This may eventually reveal that B. subtilis strains with an even higher capacity to secrete proteins with disulfide bonds can be obtained. Nevertheless, when the above described strategies were combined for the production of E. coli PhoA by B. subtilis, hardly any breakdown products of PhoA were observed, indicating that most of the PhoA was correctly folded. It will now be important to test and employ this strategy for the production of other useful proteins that contain disulfide bonds, in order to make full use of the production potential of B. subtilis.
Summary and general discussion

Sublancin 168

*B. subtilis* secretes various compounds with antimicrobial activity (Stein, 2005). One of the most interesting of these is sublancin 168. Sublancin 168 is an extremely stable lantibiotic with two disulfide bonds in addition to its methyl-lanthionine bridge. This lantibiotic exhibits bactericidal activity against other Gram-positive bacteria, including important pathogens such as *Bacillus cereus*, *Streptococcus pyogenes*, and *Staphylococcus aureus* (Hemphill et al., 1980; Paik et al., 1998). This property makes sublancin 168 a possible candidate for a new therapeutic antibiotic. The fact that its producer, *B. subtilis* 168, has also received a GRAS status from the US food and drug administration would certainly contribute to such a development. Although the genes encoding for sublancin 168 (*sunA*) and for its transporter (*sunT*) were described already several years ago (Paik et al., 1998), other factors like sublancin 168 maturation, producer immunity of *B. subtilis* 168 against its own product, and the target or mode of action of sublancin 168, has remained largely unexplored. With the work described in this thesis a more complete picture of the requirements for sublancin 168 production and function emerges.

The *sunA* and *sunT* genes are located abreast on the SPβ prophage region. Further downstream in the same operon as *sunT* lie the *bdbA*, *yolJ* and *bdbB* genes, while directly upstream of *sunA* lies the *yolF* gene. Chapter 2 documents that only the TDORs BdB and to a minor extent BdB could be involved in the formation of the disulfide bonds in sublancin 168. This refutes the previous assumptions that BdB and BdB might act as a TDOR pair in this process, or that BdB is involved. Furthermore, unpublished data showed that YolJ is essential for sublancin 168 production, possibly functioning as a glycosyltransferase (Dorenbos, Dubois, Quax and van Dijl, unpublished observations). Thus, in order to become active, the exported sublancin 168 is probably both glycosylated and oxidized.

Chapter 8 describes how *B. subtilis* maintains immunity against its own produced active sublancin 168. The YolF protein was found to be both required and sufficient for sublancin 168 immunity and was therefore renamed SunI. The, for immunity proteins, unprecedented shape of SunI – a single membrane spanning domain with the majority of the protein in the cytoplasm – seems to indicate that it belongs to a novel class of lantibiotic immunity proteins. The mechanism by which SunI confers immunity remains as yet unknown, but it is likely to interact with either sublancin 168 itself or with the target of sublancin 168.

Indications for a possible target of sublancin 168 were obtained in the studies described in chapter 9. These studies revealed that the sensitivity of target cells to sublancin 168 is inversely correlated with the NaCl concentration in the growth medium and dependent on the presence of the mechanosensitive channel protein MscL. Since MscL is likely to be open when the environmental concentrations of NaCl are low, this seemed to indicate that sublancin 168 activity is dependent on an open state of the MscL pore. Different models for the mode action of sublancin 168 can now be entertained. Sublancin 168 could function in blocking the MscL pore and thereby perhaps causing cell lysis. This
however seems unlikely since this physiological situation will be equivalent to the deletion of the \textit{mscL} gene. Clearly $\Delta mscL$ cells were not affected in viability under the conditions used to investigate the mode of action of sublancin 168 (\textit{chapter 9}). An alternative mechanism could be that MscL indirectly affects the activity of sublancin 168 when it would be involved in a separate, for sublancin 168 activity required, process. Another possibility would be that sublancin 168 requires the MscL channel for cellular entrance so that it can perform its, as yet unknown, action within the cell. This mode of action would seem to be fit with the topology of the immunity protein SunI as described in \textbf{Chapter 8}. A final possible mode of action for sublancin 168 could be that it interacts with the MscL pore and prevents it from closing. This would increase the membrane permeability for ions and/or other essential cytoplasmic compounds, which would have a severely detrimental effect on cell viability. Thus, it is conceivable that SunI provides immunity to sublancin 168 via interaction with MscL thereby antagonising the detrimental effect(s) of sublancin. Future studies that focus on mapping possible interactions between MscL, SunI and sublancin 168 should allow the investigation of these alternatives and provide further clues on the mode of action of this unique and interesting lantibiotic.