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Requirement of Signal Peptidase ComC and Thiol-Disulfide Oxidoreductase DsbA for Optimal Cell Surface Display of Pseudopilin ComGC in *Staphylococcus aureus*

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***Staphylococcus aureus* is an important Gram-positive bacterial pathogen producing many secreted and cell surface-localized virulence factors. Here we report that the staphylococcal thiol-disulfide oxidoreductase DsbA is essential for stable biogenesis of the ComGC pseudopilin. The signal peptidase ComC is indispensable for ComGC maturation and optimal cell surface exposure.**

Staphylococcus aureus is a major Gram-positive bacterial pathogen that causes a broad range of infections. To subvert its mammalian hosts, *S. aureus* relies on different virulence factors that are localized at the cell surface or secreted into the host milieu (11, 20). For export of newly synthesized virulence factors from the cytoplasm, their translocation across the membrane, and post-translocational modifications, an intricate secretion machinery has evolved (20). In recent years, the functions of many secretion machinery components of *S. aureus* have been elucidated (3, 4, 9, 13, 19, 21). Intriguingly however, for several other potential secretion machinery components, no biological functions have been described so far. For example, this applies to the extracytoplasmic thiol-disulfide oxidoreductase (TDOR) DsbA, which is known as one of the strongest bacterial TDORs (12, 15). Likewise, the role of the pseudopilin export machinery of *S. aureus* has not yet been documented. This machinery is very similar to the Com machinery for DNA binding and uptake in the related Gram-positive bacterium *Bacillus subtilis*. Interestingly, the genes coding for most Com proteins are present in the sequenced *S. aureus* strains (20). The transcription of these genes, which are organized in the *comG* and *comE* operons, is directed by the staphylococcal alternative sigma factor σ^H (17).

The biogenesis of the Com pseudopilin system has been well studied in *B. subtilis*. Among the *B. subtilis* Com proteins with orthologues in *S. aureus* are those encoded by the *comG* operon (1). Specifically, the *B. subtilis* ComGC, ComGD, ComGE, and ComGG proteins form pilin-like structures that are localized to the cytoplasmic membrane and cell wall (6–8, 22). Assembly of the pseudopilus in *B. subtilis* requires the specific signal peptidase

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TABLE 1 Primers used in this study

Primer name and purpose	Sequence (5′→3′)[a]
Construction of *S. aureus comC* mutant	
ComC-F1	CGAGATGGTCAAACATTTAAG
ComC-R1	**TCACGTCAGTCAGTCACCATGGCA**ATGACAACCTCCTTATGTAAA
ComC-F2	**TGCCATGGTGACTGACTGACGTGA**AAATTAAAGAAATGGTAA
ComC-R2	AACTGCGATGATTGCATTGGC
Construction of *S. aureus comGC* mutant	
ComGC-F1	GCTCAATAAGATAAACTTTGT
ComGC-R1	**CTACGTCAGTCAGTCACCATGGCA**ATATTAACCTCCATTATTTTA
ComGC-F2	**TGCCATGGTGACTGACTGACG**TAGAAAGCAGTCAGCATTTAC
ComGC-R2	GATTCATCATTGGTATCAATA
Construction of *S. aureus dsbA* mutant	
DsbA-F1	ATTTCTTTGGATATTTATATT
DsbA-R1	**CTACGTCAGTCAGTCACCATGGCA**AATAACTCCTATTCATAT
DsbA-F2	**TGCCATGGTGACTGACTGACG**TAGTCTTAATTGTTGAGATCA
DsbA-R2	CTTTCGTTATAGTTTTCCCAC
Construction of pCN51:comC for *S. aureus comC* overexpression	
ComC-F	CAGCCGGATCCCATAAGGAGGTTGTCATTTGGTAG (BamHI)
ComC-R	CGGAATTCCTTTAATTTTCAAAAATATACGCCTCC (EcoRI)

[a] Overlapping parts are shown in boldface. Restriction sites used for cloning are underlined (with the restriction endonuclease given in parentheses.

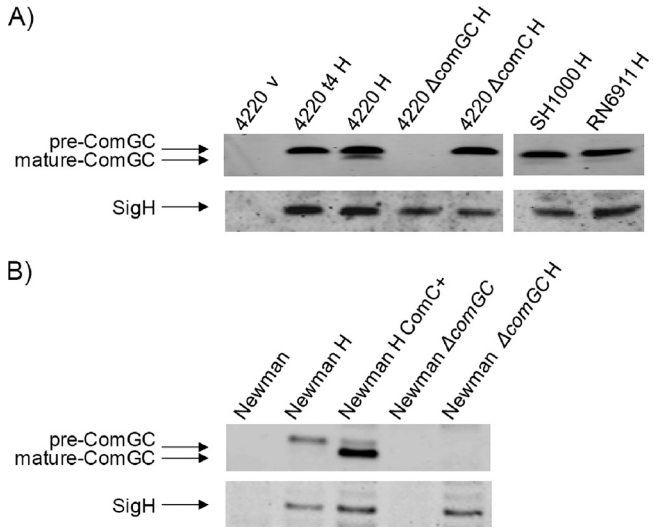

FIG 1 ComGC processing by ComC. *S. aureus* strains were grown overnight at 37°C in Luria-Bertani (LB) broth supplemented with 12.5 μg/ml chloramphenicol to select for pRIT:sigH or pRITH5 or 5 μg/ml erythromycin to select for pCN51:comC. Samples for Western blotting analyses with polyclonal rabbit antibodies against ComGC or chicken antibodies against SigH were collected after 4 h (t4) or 7 h of growth (A) or after 5 h of growth (B). Cell extracts were prepared as previously described (19). Proteins were separated by SDS-PAGE (NuPage gels; Invitrogen) and blotted onto nitrocellulose membranes (Protran; Schleicher & Schuell). Immunodetection was performed with fluorescent secondary antibodies (IRDye 700 CW goat anti-rabbit and IRDye 800 goat anti-chicken; Li-Cor) in combination with the Odyssey infrared imaging system (LiCor Biosciences). The chromosomal *comGC* or *comC* genes were deleted from *S. aureus* strain RN4220 or Newman Δ*spa* Δ*sbi* (19) as previously described (2, 14). The primers used for strain and plasmid constructions are listed in Table 1. Lanes relating to strains that carry pRIT:sigH for σ^H production are indicated by "H"; lanes relating to control strains with the empty vector pRIT5H that do not produce σ^H are indicated by "v"; lanes relating to strains that carry pCN51:comC (5) for ComC production are indicated by "ComC+."

ComC, which processes the N-terminal signal peptides of ComG proteins upon membrane translocation (6, 22, 23). Furthermore, stability of the *B. subtilis* ComGC pseudopilin requires posttranslocational TDOR-mediated disulfide bond formation (6, 10, 15, 16).

ComGC of *S. aureus* is processed by ComC and stabilized by DsbA. To study the processing and stability of *S. aureus* ComGC, the expression of the *com* genes was induced through constitutive expression of *sigH* from plasmid pRIT:SigH (17). The primers used for strain and plasmid constructions are listed in Table 1. As shown with specific polyclonal antibodies raised against ComGC, exponentially growing cells of the *S. aureus* strains RN4220, SH1000, RN6911, and Newman produced only the precursor form of this pseudopilin (Fig. 1). ComGC production depended strictly on ectopic expression of σ^H (Fig. 1). Notably, relatively small amounts of mature ComGC were detectable when the investigated strains were grown to stationary phase (Fig. 1A). This inefficient ComGC processing was due to limited ComC signal peptidase activity, as shown by ComC overexpression from plasmid pCN51:comC. This resulted in close-to-complete ComGC processing (Fig. 1B). Conversely, ComGC processing in the post-exponential growth phase was completely abolished by a *comC* deletion. Consistent with these observations, no *comC* expression was detectable in exponentially growing *S. aureus* cells, and low-level *comC* transcription was detectable in the late stationary growth phase (data not shown). Together, these findings show that ComC is the signal peptidase needed for ComGC processing and that the investigated strains produce limiting amounts of ComC under the tested conditions.

To investigate whether the stability of *S. aureus* ComGC depends on TDOR activity, the production of this protein was analyzed in strain RN4220 lacking the *dsbA* gene. Western blotting analyses showed that ComGC was barely detectable in cells lacking DsbA. This effect did not relate to possible changes in the σ^H levels, which remained unaltered in the *dsbA* mutant (Fig. 2, lower panel). These observations indicate that the intramolecular disulfide bond of *B. subtilis* ComGC is conserved in *S. aureus* ComGC and that the formation of this disulfide bond between Cys46 and Cys87 of *S. aureus* ComGC is catalyzed by DsbA. Furthermore, this disulfide bond, which is positioned within the predicted extracytoplasmic domain of *S. aureus* ComGC, would be necessary to stabilize ComGC upon export from the cytoplasm. To test this idea, the reducing agent β-mercaptoethanol was added to the growth medium at concentrations up to 2.5 mM, which is the highest concentration of β-mercaptoethanol that can be added to the cells without affecting growth and cell viability (data not shown). Clearly, in the presence of 2.5 mM β-mercaptoethanol, ComGC was barely detectable (Fig. 2, upper panels), whereas σ^H production remained unaffected (Fig. 2, lower panels). The simplest explanation for these observations is that the TDOR activity of DsbA is required for disulfide bond formation in ComGC and

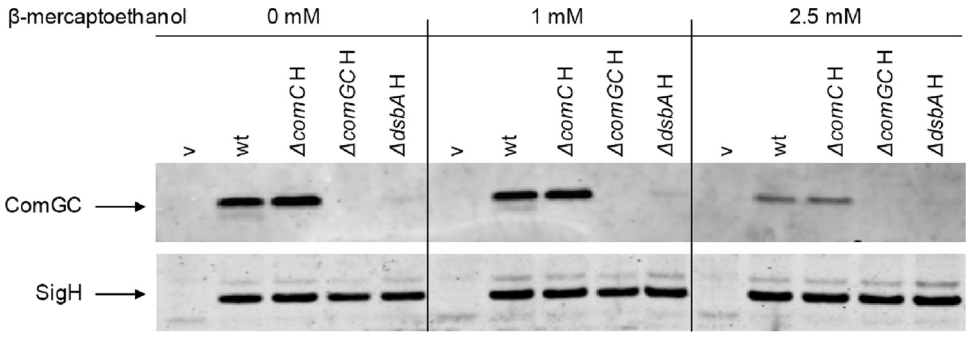

FIG 2 ComGC stabilization by DsbA. *S. aureus* strains were grown for 7 h in LB broth as described in Fig. 1 in the presence or absence of β-mercaptoethanol (final concentration, 1 or 2.5 mM). The preparation of cell extracts, SDS-PAGE, and Western blotting with specific antibodies against ComGC or σ^H were performed as described in the legend to Fig. 1. The chromosomal *dsbA* gene was deleted from *S. aureus* strain RN4220 as previously described (2, 14). Primers used for strain construction are listed in Table 1. Lanes relating to strains that carry pRIT:sigH for σ^H production are indicated by "H"; lanes relating to control strains with the empty vector pRIT5H that do not produce σ^H are indicated by "v."

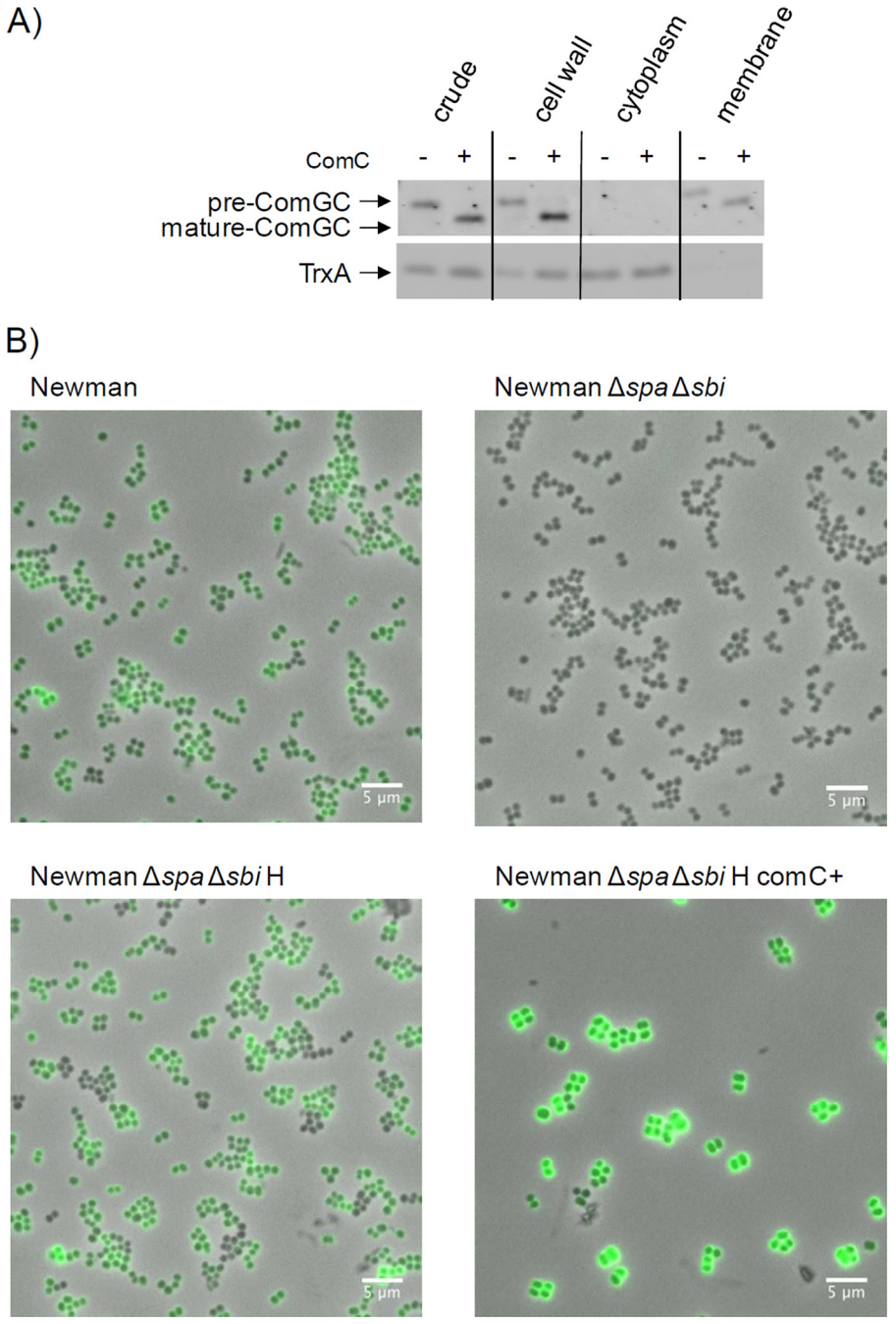

FIG 3 ComGC localizes to the membrane, cell wall, and cell surface of *S. aureus*. (A) To determine the subcellular localization of ComGC in *S. aureus* RN4220/pRIT:sigH or *S. aureus* RN4220/pRIT:sigH containing pCN51:comC, the cells were grown in LB broth for 5 h, collected by centrifugation, and incubated for 1 h at 37°C in protoplast buffer (50 mM Tris-HCl [pH 7.6], 0.145 M NaCl, 30% sucrose, 0.01% DNase, and EDTA-free Complete protease inhibitors [Roche]). The cell wall fraction (i.e., protoplast supernatant) was obtained by centrifugation (20 min, 3,000 × *g*, 4°C). Protoplasts were disrupted by osmotic shock in 0.05 M Tris-HCl (pH 7.6) with 30 min of incubation on ice and vortexing at 5-min intervals. Cytosolic and solubilized membrane proteins were collected as previously described (24). SDS-PAGE and Western blotting with specific antibodies against ComGC or the cytoplasmic control protein TrxA were performed as described in the legend to Fig. 1. (B) Cell surface exposure of ComGC was assessed in *S. aureus* Newman Δ*spa* Δ*sbi* or the parental strain (Newman) by immunofluorescence microcopy. For this purpose, cells were grown for 5 h in LB broth, and 1 unit of cells (by optical density at 600 nm) was collected by centrifugation (8,000 rpm, 5 min, 4°C). The cell pellet was resuspended in phosphate-buffered saline-Tween 20 (PBST) plus 2% bovine serum albumin (BSA) and incubated for 10 min on ice. Next, the cells were incubated for 60 min with ComGC-specific polyclonal rabbit antibodies (1:400 in PBST plus 1% BSA). Unbound antibodies were removed by three washes in PBST, and cell-bound ComGC antibodies were visualized using goat-anti-rabbit Alexa Fluor 488 antibodies (Life Technologies) and a Leica DM5500 B microscope. The overlay of phase-contrast and fluorescence microscopy images was done with imageJ. The strains containing pRIT:sigH for σ^H production are indicated by "H"; the strain containing pCN51:comC for ComC production is indicated by "ComC+." The magnification is indicated by scale bars.

that this disulfide bond is essential for ComGC stability. Nevertheless, it is possible that DsbA is indirectly involved in the stabilization of ComGC. To our knowledge, this is the first report describing a biological function for DsbA in *S. aureus*.

ComGC localizes to the membrane, cell wall, and cell surface of *S. aureus*. To determine the localization of ComGC, cells of *S. aureus* strain RN4220 or strain RN4220 overproducing ComC were subjected to subcellular fractionation. Cells were first protoplasted. Next, the protoplasts were separated from liberated cell wall proteins (i.e., the cell wall fraction) by centrifugation. The collected protoplasts were then disrupted by osmotic shock, and cytosolic proteins were separated from the membranes by ultracentrifugation as previously described (24). Proteins in all collected fractions were separated by SDS-PAGE, and the presence of ComGC and thioredoxin A (TrxA) in each fraction was analyzed by Western blotting with specific antibodies (Fig. 3A). TrxA was used as a cytoplasmic control protein. This analysis showed that both pre-ComGC and mature ComGC from *S. aureus* localize to the cytoplasmic membrane and cell wall. This dual localization is consistent with the localization of the homologous protein in *B. subtilis*. Furthermore, in *S. aureus* cells overproducing the ComC protein, we observed slightly increased amounts of ComGC in the cell wall fraction compared to cells of the parental control strain that do not produce ComC under the tested conditions. Next, we investigated by immunofluorescence microscopy whether ComGC is detectable on the cell surface. For this purpose, we employed cells of strain Newman lacking the IgG-binding proteins Spa and Sbi, which displayed negligible background fluorescence (Fig. 3B; compare panels for the *spa sbi* mutant and the parental strain Newman). Importantly, σ^H-producing cells showed elevated levels of immune fluorescence, and strongly enhanced immune fluorescence was observed when the signal peptidase ComC was overexpressed together with σ^H. These observations are consistent with the view that the signal peptide of ComGC facilitates membrane translocation and exposure of ComGC to the cell wall, irrespective of signal peptide processing by ComC. Enhanced signal peptide processing upon ComC overproduction would then allow more of the translocated mature ComGC to penetrate the cell wall and to become exposed at the cell surface. These findings thus show that ComC-dependent processing of ComGC is important for optimal cell surface exposure of ComGC. It should be noted that, for unknown reasons, cells overproducing ComC have a larger diameter, which seems to relate mostly to a thickened cell wall (Fig. 3B).

In summary, we show that biogenesis of the pseudopilin ComGC of *S. aureus* requires the TDOR DsbA for stability and the signal peptidase ComC for precursor maturation and cell surface exposure. This is thus the first report in which biological functions are demonstrated for *S. aureus* DsbA and ComC. In *B. subtilis*, the Com system is needed for DNA uptake during genetic competence. Whether this is also true in *S. aureus* remains to be demonstrated, but natural competence has been reported for *S. aureus* (18). Our present findings suggest that expression of *comC* could be a limiting factor in competence development, even if *S. aureus* cells overproduce σ^H for expression of other *com* genes.

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REFERENCES

1. **Albano M, Breitling R, Dubnau DA.** 1989. Nucleotide sequence and genetic organization of the *Bacillus subtilis comG* operon. J. Bacteriol. **171**: 5386–5404.
2. **Arnaud M, Chastanet A, Debarbouille M.** 2004. New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, gram-positive bacteria. Appl. Environ. Microbiol. **70**:6887–6891.
3. **Biswas L, et al.** 2009. Role of the twin-arginine translocation pathway in *Staphylococcus*. J. Bacteriol. **191**:5921–5929.
4. **Burts ML, Williams WA, DeBord K, Missiakas DM.** 2005. EsxA and EsxB are secreted by an ESAT-6-like system that is required for the pathogenesis of *Staphylococcus aureus* infections. Proc. Natl. Acad. Sci. U. S. A. **102**:1169–1174.
5. **Charpentier E, et al.** 2004. Novel cassette-based shuttle vector system for gram-positive bacteria. Appl. Environ. Microbiol. **70**:6076–6085.
6. **Chen I, Provvedi R, Dubnau D.** 2006. A macromolecular complex formed by a pilin-like protein in competent *Bacillus subtilis*. J. Biol. Chem. **281**:21720–21727.
7. **Chung YS, Breidt F, Dubnau D.** 1998. Cell surface localization and processing of the ComG proteins, required for DNA binding during transformation of *Bacillus subtilis*. Mol. Microbiol. **29**:905–913.
8. **Chung YS, Dubnau D.** 1998. All seven *comG* open reading frames are required for DNA binding during transformation of competent *Bacillus subtilis*. J. Bacteriol. **180**:41–45.
9. **Cregg KM, Wilding I, Black MT.** 1996. Molecular cloning and expression of the *spsB* gene encoding an essential type I signal peptidase from *Staphylococcus aureus*. J. Bacteriol. **178**:5712–5718.
10. **Draskovic I, Dubnau D.** 2005. Biogenesis of a putative channel protein, ComEC, required for DNA uptake: membrane topology, oligomerization and formation of disulphide bonds. Mol. Microbiol. **55**:881–896.
11. **Dreisbach A, van Dijl JM, Buist G.** 2011. The cell surface proteome of *Staphylococcus aureus*. Proteomics **11**:3154–3168.
12. **Dumoulin A, Grauschopf U, Bischoff M, Thony-Meyer L, Berger-Bachi B.** 2005. *Staphylococcus aureus* DsbA is a membrane-bound lipoprotein with thiol-disulfide oxidoreductase activity. Arch. Microbiol. **184**:117–128.
13. **Jongbloed JD, van der Ploeg R, van Dijl JM.** 2006. Bifunctional TatA subunits in minimal Tat protein translocases. Trends Microbiol. **14**:2–4.
14. **Kouwen TR, et al.** 2009. The large mechanosensitive channel MscL determines bacterial susceptibility to the bacteriocin sublancin 168. Antimicrob. Agents Chemother. **53**:4702–4711.
15. **Kouwen TR, et al.** 2007. Thiol-disulphide oxidoreductase modules in the low-GC Gram-positive bacteria. Mol. Microbiol. **64**:984–999.
16. **Meima R, et al.** 2002. The bdbDC operon of *Bacillus subtilis* encodes thiol-disulfide oxidoreductases required for competence development. J. Biol. Chem. **277**:6994–7001.
17. **Morikawa K, et al.** 2003. A new staphylococcal sigma factor in the conserved gene cassette: functional significance and implication for the evolutionary processes. Genes Cells **8**:699–712.
18. **Rudin L, Sjostrom JE, Lindberg M, Philipson L.** 1974. Factors affecting competence for transformation in *Staphylococcus aureus*. J. Bacteriol. **118**: 155–164.
19. **Sibbald MJ, et al.** 2010. Synthetic effects of *secG* and *secY2* mutations on exoproteome biogenesis in *Staphylococcus aureus*. J. Bacteriol. **192**:3788–3800.
20. **Sibbald MJ, et al.** 2006. Mapping the pathways to staphylococcal pathogenesis by comparative secretomics. Microbiol. Mol. Biol. Rev. **70**:755–788.
21. **Stoll H, Dengjel J, Nerz C, Gotz F.** 2005. *Staphylococcus aureus* deficient in lipidation of prelipoproteins is attenuated in growth and immune activation. Infect. Immun. **73**:2411–2423.
22. **Tjalsma H, et al.** 2004. Proteomics of protein secretion by *Bacillus subtilis*: separating the "secrets" of the secretome. Microbiol. Mol. Biol. Rev. **68**: 207–233.
23. **Tjalsma H, Bolhuis A, Jongbloed JD, Bron S, van Dijl JM.** 2000. Signal peptide-dependent protein transport in *Bacillus subtilis*: a genome-based survey of the secretome. Microbiol. Mol. Biol. Rev. **64**:515–547.
24. **Zweers JC, Wiegert T, van Dijl JM.** 2009. Stress-responsive systems set specific limits to the overproduction of membrane proteins in *Bacillus subtilis*. Appl. Environ. Microbiol. **75**:7356–7364.