Biochemistry (Moscow), Vol. 67, No. 11, 2002, pp. 1207-1216. Translated from Biokhimiya, Vol. 67, No. 11, 2002, pp. 1461-1473.

REVIEW

Late Stages of Protein Secretion in Bacilli

M. R. Sharipova

Department of Microbiology, Kazan State University, ul. Kremlevskaya 18, Kazan, 420008 Russia;
E-mail: Margarita.Sharipova@ksu.ru

Received November 9, 2001
Revision received February 26, 2002

Abstract—This review highlights the later stages of protein secretion in bacilli, which include protein release from the membrane and their translocation through the cell wall. Mechanisms of release of secreted polypeptides into the medium differ in Gram-positive and Gram-negative bacteria due to different structure of their cell envelope. Exogenous factors including molecular chaperons that can influence polypeptide folding may be also involved in later stages of protein secretion in bacilli. In Gram-positive bacteria protein secretion also depends on structural components of the cell wall. Certain evidence exists that maintenance of the secretory function is important for normal development of the sporulation process in these bacteria.

Key words: bacilli, secretion, cell wall, metal cations, PrsA-lipoprotein, FtsH-protease, sporulation

SECRETION. GENERAL INFORMATION

Secretion is a rather complex multistage process, including translocation of protein molecules through the cytoplasmic membrane from the cytoplasm (where they have been synthesized) to a specific destination to outer cellular compartments or the culture medium. During this secretion proteins pass through all stages of their biogenesis; they undergo conversion of inactive precursors into active macromolecules. The secreted proteins contain an additional N-terminal sequence known as a signal peptide. This signal peptide promotes the export process. It interacts with membrane protein and cytoplasmic protein components, which prevent folding of the secreted protein and maintain conformation of this protein favoring its transmembrane translocation. A special protein complex, translocase, catalyzes transmembrane protein translocation. During protein translocation a special signal peptidase located on the outer surface of the cytoplasmic membrane removes the signal peptide and the protein is released from the membrane. Some translocated proteins that underwent processing remain in the membrane-bound state until complete folding. This stage may involve specific protein chaperons, which in contrast to cytoplasmic unfoldases facilitate formation of tertiary and even quaternary structures.

Transmembrane protein translocation is a basic cell process, which shares significant similarities among Gram-positive and Gram-negative bacteria and also eukaryotes. There are several excellent reviews by M. A. Nesmeyanova [1-3] and others [4, 5] which highlight molecular mechanisms of protein secretion through the bacterial membrane including co-translational and post-translational translocation, the concept of coupling of protein translocation with transmembrane phospholipid movement, and the energetics of these processes.

In spite of certain similarity of protein translocation through cytoplasmic membrane, differences in structure of cell envelope of Gram-positive and Gram-negative bacteria determine different routes of protein secretion into the external medium after protein release from the membrane. In bacilli secreted proteins initially cross the cytoplasmic membrane and then during translocation through the cell wall and release into extracellular medium they undergo post-translational modifications. In these bacteria the main differences in protein secretion are related to the later stages of protein (polypeptide) export.

Here we consider molecular mechanisms of terminal stages of protein secretion in Gram-positive bacteria.

Bacilli secrete more proteins than other microorganisms and this implies high efficacy of their secretory apparatus. Many bacillus strains are employed in industry for production of amylases and proteases and their annual production is about 1000 tons [6]. Such high productivity of the industrial strains is achieved by employment of classic methods of selection of cultivation media and optimization of promoter functioning [7]. Bacilli attract much attention as potential producers of heterologic proteins. They secrete proteins into the cultivation medium and this fact significantly simplifies isolation of these proteins. The genome of these

0006-2979/02/6711-1207$27.00 ©2002 MAIK "Nauka/Interperiodica"

microorganisms is completely sequenced [8] and a wide range of gene engineering and biotechnological methods is available now for solution of numerous problems. Nevertheless, studies have revealed rather ineffective secretion of heterologic (especially eukaryotic) proteins by the recombinant strains and this limits potential use of these microorganisms. One possible reason of such ineffectiveness may be related to problems in translocation of certain polypeptides at late stages of protein export. This may result in imbalance between processes of their translation and translocation, accumulation of inactive protein precursors, which cannot acquire final conformation and therefore are cleaved by cellular proteases [5, 7]. Certain evidence exists that differences in production of extracellular proteins by *Bacillus subtilis* are consistent with different stability of corresponding mRNAs [9].

Recently, several rate-limiting steps of protein secretion have been identified in *B. subtilis*; they are related to the export mechanism and also to properties of the secreted polypeptides [10]. Heterologic proteins may form insoluble complexes in the cytoplasm due to absence of chaperons required for their proper folding [11]. Incorrect processing of heterologic protein precursor may also limit the secretion process [12]. Specific *B. subtilis* folding chaperons (PrsA and others) bound to the outer surface of membrane may also limit secretion of some heterologic proteins [7]. The cell wall of bacilli may also be an impermeable barrier for release of eukaryotic proteins [13]. In some cases low yield of a sought protein may be attributed to its high sensitivity to proteases which bacilli secrete into the cultivation medium (some proteases are bound to the cell surface) [14, 15].

The effectiveness of secretion which depends on properties of the secreted protein is related to later stages of the protein export: processing and protein translocation through the cell wall [10]. This was demonstrated during comparative study of *B. subtilis* secretion of four heterologic proteins: *Bacillus licheniformis* α-amylase (AmyL, EC 3.2.1.1, molecular mass of 55 kD), *Escherichia coli* β-lactamase (Bla, EC 3.5.2.6, 30 kD), human pancreatic α-amylase (HPA, EC 3.2.1.1, 50 kD), and a single chain anti-lysozyme antibody (SCA-Lys) [10]. The protein secretion was investigated under identical conditions of expression under the same signal peptide. Use of radioactive label, fluorescent probes, and immunoblotting revealed that all heterologic proteins were normally transported through the cytoplasmic membrane of *B. subtilis*. Effectiveness of expression was determined by steps which followed after the translocation. The rate-limiting steps in secretion of Bla, AmyL, and SCA-Lys were processing, translocation through the cell wall, and proteolysis. After the transmembrane translocation HPA was accumulated in the cell due to lack of folding of this protein. (Mature conformation of this protein molecule results from formation of three disulfide bonds.) It was demonstrated that *B. subtilis* oxidoreductases required for effective secretion of disulfide bond containing proteins did not operate correctly in the case of HPA. These studies revealed an obvious need for elucidation of the rate-limiting steps in the secretion of heterologic proteins and their correction by gene engineering manipulations with host strains.

Sec-PROTEINS

Secreted proteins are transported through the bacterial cytoplasmic membrane by preprotein translocase; several integral membrane proteins, SecY, SecE, SecG, and SecDF, are involved in its formation [16-19]. Peripheral membrane protein SecA plays a key role in the initiation of protein translocation through the cytoplasmic membrane; this protein specifically interacts with the signal sequence and also with certain sites of the mature protein [20]. SecA possess ATPase activity which is stimulated by high affinity interaction with precursors of the secreted proteins [21]. Variations in their structure are crucial for the interaction with SecA protein. Comparative analysis of secretion of *B. subtilis* levansaccharase (EC 2.4.1.10, 50 kD) and α-amylase (EC 3.2.1.1, 68 kD) revealed significant differences in binding of precursors of these proteins with SecA during exponential growth when the cellular level of SecA is subjected to marked variations [22]. Secretion of levansaccharase correlated with the cell level of SecA, whereas α-amylase secretion increased only in the case of significant reduction of SecA protein level. The authors concluded that the difference of protein secretion induced by changes of SecA content corresponds to the difference in the affinity of precursors of the secreted proteins to SecA. In *E. coli* cells exoprotein precursors are fixed in the state favorable for translocation; this involves unfoldase SecB. *B. subtilis* lacks any homolog of unfoldase SecB [22], which was not found even after analysis of the full genome sequence [8]. Perhaps, during maturation of proteins in bacilli chaperone function may be attributed to structural domain of the precursor (propeptide) located between signal peptide and the protein sequence corresponding to the mature protein [5]. It is also possible that specific cytoplasmic factors (e.g., pH, metal ion concentrations, etc.) may mimic chaperone function.

This suggests the importance of information accumulated in the precursor sequence, including sites of the mature polypeptide sequence. Using *E. coli* alkaline phosphatase (EC 3.1.3.1, 80 kD) it was shown that effectiveness of secretion of mutant proteins with amino acid substitutions in the N-terminal region of the mature polypeptide chain depends on their locations: the closer the amino acid substitution is located to the signal peptide the less effective secretion is [23].

PROCESSING

Two signal peptidases type I (SipS and SipT) and one signal peptidase type II (Lsp) are involved in processing of secreted proteins of *B. subtilis*. SipS and SipT remove signal polypeptide of proproteins and Lsp cleaves signal peptide of polyprotein [24, 25]. Processing of spore-specific TasA protein requires special SipW peptidase which was recently identified [26]. Two recently found constitutive signal peptidases, SipU and SipV, are expressed at low level over all phases of *B. subtilis* growth [24, 25].

The two component regulatory system DegS–DegU controls SipS transcription; this means that SipS expression is regulated by the same factors as the expression of most secreted hydrolases of *B. subtilis* [12]. In contrast to *E. coli* signal peptidase lack of SipS is not critical for protein secretion and survival of *B. subtilis*. In this case a rate of processing of some protein reduces whereas the rate of processing of *Bacillus amyloliquefaciens* α-amylase (EC 3.2.1.1, 50 kD) and two unidentified proteins increases. Thus, substrate specificity of the signal peptidase determining the rate of signal peptide processing is one of the main factors determining protein secretion in bacilli [27].

After processing, signal peptides are cleaved in the membrane or cytoplasm. Oligopeptides formed during such cleavage may also have signal function [28]. These results suggest an important role of peptidases involved in hydrolysis of signal peptides. In was shown that the genome of *B. subtilis* contains two genes, encoding SppA and TepA proteins, which are homologous to peptidase A hydrolyzing signal peptides of *E. coli* [28]. Based on homology to known proteases we may suggest that TepA and SppA peptidases are required for specific degradation of signal peptides and the hydrolytic products restrain protein translocation [28]. These peptidases have different subcellular distribution: SppA is an integral membrane protein of *B. subtilis*, whereas TepA is located in the cytoplasm. Loss of both enzymes significantly influences membrane translocation of exoprotein precursors in bacilli.

EFFECT OF EXOGENOUS EFFECTORS ON PROTEIN SECRETION

Most secreted proteins of bacilli are folded into mature conformation on the outer surface of the cytoplasmic membrane. The kinetics of protein folding plays an important role at the stage of protein release into the medium. Comparative study of gene expression of *B. subtilis* levanase (SacC) (EC 3.2.1.26, 75 kD) under control of inducible promoter and signal sequence of levansaccharase (SacR) in *B. subtilis* degU32(Hy) strain and also genes encoding *B. subtilis* levansaccharase and α-amylase revealed that the rate of protein translocation through the cell wall correlated with the rate of folding of corresponding proteins irrespectively of the size of the molecules [29].

In *E. coli* effectors restraining protein folding (unfoldases) have been identified in the cytoplasm. They maintain the state of protein competent for its export. Effectors localized on the outer surface of the cytoplasmic membrane of *B. subtilis* promoting protein folding to native conformation cause irreversible translocation. Different effectors are involved in protein folding.

PrsA protein. Specific proteins, foldases, constitute one group of such effectors. The following foldases are involved into formation of tertiary and quaternary structures of secreted proteins: BdbB, BdbC, BlaP, and PrsA [7, 10, 30]. The latter, membrane bound lipoprotein PrsA is a peptidyl-prolyl-*cis/trans*-isomerase (EC 5.2.1.8). Its protein domain is located at the outer surface of the cytoplasmic membrane and facing to the cell wall [7, 31]. Activation of PrsA was observed during the posttranslational phase of secretion; PrsA is required for secretion of (almost) all exoproteins [7, 32].

Membrane-bound PrsA functions as extra-cytoplasmic chaperone during folding of secreted proteins after translocation through the membrane. Increase of PrsA synthesis by using multi-copied plasmid in *B. subtilis* cells significantly caused 2-6-fold increase of output of α-amylases and proteases into the cultivation medium [7]. In PrsA-deficient strains of *B. subtilis* accumulation of exoproteins including α-amylase and subtilisin (EC 3.4.21.62, 27.5 kD) sharply reduced [32]. Study of inducible expression of the chimeric protein subtilisin–alkaline phosphatase in PrsA3 mutants of *B. subtilis* revealed complete loss of its activity [32]. This suggests that *in vivo* PrsA acts as a chaperone, operating at later stages of secretion; it promotes formation of a mature conformation of the secreted proteins at the stage of their release into the cultivation medium. A direct relation exists between the number of PrsA molecules in wild types of *B. subtilis* (20,000) and the number of secreted molecules of α-amylase [33]. Sharp reduction of PrsA content in *B. subtilis* cells results in the change of cell morphology and subsequent cell death. Lack of PrsA-like proteins in *E. coli* cells raises reasonable question about the possible functioning of these lipoproteins as specific chaperones in the cell wall of Gram-positive bacteria.

Computer analysis revealed that the genome of *B. subtilis* contains about 300 genes encoding secreted proteins possessing N-terminal signal peptide [25]; 114 of them are lipoproteins bound to cytoplasmic membrane. The importance of lipoproteins for cell homeostasis of *B. subtilis* was demonstrated by using signal peptidase II-deficient mutants [25]. Processing of PrsA was essential for survival of *B. subtilis* cells cultivated at low or high temperatures (but inessential for bacterial growth at 37°C), for the development of competent state of the cells, for normal sporulation, and subsequent spore ger-

mination. Cells lacking signal peptidase II were characterized by low rate of accumulation of lipid-modified precursor of PrsA. Secretion of α-amylase was significantly impaired in these mutants. Lack of alternative lipoprotein splicing by signal peptidases type I was quite unexpected for these bacilli.

In *B. subtilis* lipid modification is controlled by the *lgt*-gene [30]; *lgt*-mutants were totally viable and unmodified forms of PrsA and BlaP were functionally active. However, they were unable to bind to the membrane and be released into the cultivation medium. This was accompanied by reduction of total amount of cell-bound lipoproteins. Low level of PrsA lipoprotein caused impairments in total protein secretion (*lgt*-mutant phenotype).

Metal cations. Metal cations represent the other group of exogenous folding effectors in bacilli. Membrane of *B. subtilis* is surrounded by rather thick cell wall (from 10 to 50 nm) of heteropolymeric matrix that consists of peptidoglycan and teichoic acids [34]. Carboxyl groups of peptidoglycan and teichoic acid phosphate groups determine anionic nature of *B. subtilis* cell wall, which can bind positively charged cations, Ca^{2+}, Fe^{3+}, etc. [34, 35]. During export into the cultivation medium secreted proteins have to cross the cell wall (as the terminal stage of secretion) and acquire mature conformation in this compartment before release into the medium.

It was suggested that the difference in concentration of metal ions (Fe^{3+} and Ca^{2+}) on the inner and outer surfaces of the cytoplasmic membrane of bacilli is important for folding reaction of secreted polypeptides possessing metal-binding sites in the mature structure [36]. The rate and effectiveness of metal-dependent folding is crucial for production of these proteins. In this case the driving force for protein translocation corresponds to chemical asymmetry of certain effector between *cis*- and *trans*-sides of the membrane; this force represents the result of coupling of two processes: protein folding and its translocation through cytoplasmic membrane [37].

In tertiary and quaternary structures of many bacilli secreted proteins Ca^{2+}-dependent sites have been identified. For example, BPN′ (or Carlsberg) subtilisin (EC 3.4.21.62, 27.5 kD) possesses two different Ca^{2+}-binding sites, characterized by high (K_a 10^8 M^{-1}) and low (K_a 32 M^{-1}) affinity to this cation [38]. The low affinity binding site can bind Ca^{2+} only in Ca^{2+}-enriched medium (>40 mM), which was not found in the natural environment. Thus, low affinity binding sites may be involved into secretion only when protein are in the cell wall layer, which is characterized by rather high concentration of Ca^{2+}. The fact that Ca^{2+} is immobilized on polymers of the cell wall seems to favor conformation transition of secreted protein due to higher affinity of immobilized calcium for secreted proteins than that of the free ion [39]. Such secretion may also occur via chaperone-independent export.

Extracellular levansaccharase of *B. subtilis* (SacB) was employed as the first model for such studies [40]. The gene encoding this protein does not have the nucleotide sequence responsible for the propeptide region localized between signal peptide and the mature structure, which functions as endogenous chaperone in bacilli and is involved in coupling and secretion [5]. The low affinity Ca^{2+} binding site (K_a $0.8 \cdot 10^3$ M^{-1}, at pH 7.0) was identified in the mature levansaccharase structure. Amino acid substitution in this region (Thr263Ile) changed the affinity for Ca^{2+} and reduced effectiveness of the polypeptide secretion [41]. The cell wall of *B. subtilis* may concentrate (up to 100-120-fold) Ca^{2+}. This provides high concentration of Ca^{2+} (>2 mM) at the external side of cytoplasmic membrane. Such membrane microenvironment provides conformational transition of levansaccharase molecule during secretion and leads to formation of mature extracellular protein. So it was concluded that Ca^{2+} plays the key role at the last stage of secretion of this protein in the medium [40].

Secretion of *B. subtilis* levansaccharase occurs in two stages [42]. The first stage includes proteolytic cleavage of the N-terminal signal followed by formation of membrane bound enzyme. The second stage is the secretion limiting stage consisting of release of this enzyme from the membrane into the medium, and this is accompanied by conformational changes in the exported protein.

The levansaccharase unfolding-folding transition depends on the enzyme ionization [41]. During cultivation of *B. subtilis* at pH < 6.0 the concentration of H^+ at the outer surface of cytoplasmic membrane may exceed corresponding H^+ concentration by 200-fold [43]. The effect of transmembrane ΔpH on *B. subtilis* protein secretion was described earlier [44], but the mechanism of this effect remained unclear. Now certain evidence exists that H^+ and Ca^{2+} are involved in protein folding coupled to its secretion. At 30°C in the absence of Ca^{2+} *in vitro*, relatively small shift of pH value from 7.4 to 6.0 [45] resulted in complete folding of levansaccharase which was due to rapid shift between equilibrium of unfolding–folding states. High Ca^{2+} concentrations (>5 mM) acted as the catalyst of protein molecule folding at pH > 7.0. *In vivo* experiments employing label at 30°C revealed that in Ca^{2+}-free medium the rate of the second step of levansaccharase secretion depended on pH of the medium. In acidic medium (at pH 5.8) the secretion was more effective. At pH > 7.0 the presence of Ca^{2+} was more essential for the secretion process. At higher temperature (48°C) both factors, low pH and Ca^{2+} concentration values are required for effective secretion. Levansaccharase with altered affinity for Ca^{2+} (Thr263Ile) was actively secreted only in acidic medium (i.e., under Ca^{2+}-independent conditions).

Thus, it was shown that the concentration of the external effectors (H^+ or Ca^{2+}), which was maintained between *cis/trans* sides due to properties of bacterial cell

wall, plays the key role at later stages of protein secretion in bacilli.

The extracellular *B. subtilis* α-amylase is another example of protein secretion [46]. The α-amylase secretion pathway is similar to that of levansaccharase [45]. Both pathways include a cleavage of signal peptide followed by the formation of the membrane-bound form [47] and subsequent polypeptide release into the medium. The latter depended on the conformational rearrangement of the protein molecule.

The comparison of effectiveness of secretion of these proteins by *B. subtilis* revealed that the enzymes exhibit different affinity with respect to Ca^{2+} [45, 46]. The main difference in the secretion of α-amylase and levansaccharase consists in kinetics of these processes: the rate of processing of signal peptide was higher in the case of α-amylase, whereas the release of α-amylase was two times higher than that of levansaccharase. The use of fluorescent methods revealed the intermediary folding product at the stage of release of *B. subtilis* α-amylase from the membrane into the cultivation medium [47]. This intermediate was inactive, and it was characterized by a relative stability to proteases. It was shown that the interaction underlying cation transfer from a ligand to ligand (e.g., teichoic acid to the protein) is more thermodynamically favorable than the binding of fully hydrated cations from the solution [39]. The transition of a protein molecule into mature conformation requires the presence of Ca^{2+}. (Although, the metal ions were not preserved in the associated state with the mature protein form.) Production of the mutant *B. subtilis* α-amylase, characterized by increased total positive charge of the protein globule, was significantly lower than in the case of the initial strain. The folding rate of the mutant protein exhibited the pronounced dependence on the presence of Ca^{2+} in the medium. The increase of Ca^{2+} concentration caused an increase in the enzyme production [48]. To determine the effect of total charge of the protein molecule on later stages of *B. licheniformis* α-amylase secretion mutant protein with p*I* of 5.0 and 10.0 and also native protein with p*I* of 7.0 were used [49]. Physicochemical characteristics and enzymatic properties of these enzymes remained virtually preserved. It was shown that the positively charged protein interacts with the components of the cell wall and this significantly influences the pathway for its release into the outer environment.

Thus, the formation of native conformation of secreted proteins occurs in the outer compartments of bacilli and this is mainly determined by environmental conditions. Their changes cause the formation of products with incomplete folding which are cleaved by proteases. This process coupled to the protein release into the medium through the cell wall matrix is the rate-limiting step of secretion. Some processes of uncontrolled secretion are exceptions. In some cases *B. subtilis* protoplasts were used in experiments and the disruption of their cell wall resulted in significant reduction of α-amylase secretion [46]. Another fate (under stress conditions) was the blockade of secretion of other proteins by overexpression of stress proteins [50, 51].

We studied the secretion of various hydrolytic enzymes by *Bacillus intermedius*. One of selected enzymes, guanyl-specific ribonuclease (EC 3.1.4.23, 12.3 kD), contains a large number of hydrophobic amino acid residues and in the absence of cysteine residues this provides high resistance of the enzyme over a wide range of pH and temperature [52]. The subtilisin-like thiol-dependent serine protease (EC 3.4.21.62, 32.5 kD) is characterized by increased content of Asx and Glx residues; it has 1-3 residues of half-cystines per molecule. This provides the maintenance of relatively stable conformation. Calcium ions are involved in stabilization of the protein molecule [53]. The alkaline phosphatase (EC 3.1.3.1, 46 kD) molecule lacks free SH groups or S–S bonds; this enzyme is characterized by relative stability [54]. In contrast to RNase, which is stable in the range of pH 2-10 and resistant to heating up to 90°C the protease is resistant (pH 6.3-10.5, and there was 10% inactivation during exposure for 30 min at 55°C), whereas phosphatase is a labile protein (pH 8-10, and there was 60% inactivation during 30 min at 55°C).

There was a coincidence between the dynamics of synthesis of polyadenylated mRNA in *B. intermedius* cells and dynamics of extracellular enzyme synthesis (RNase and phosphatase) in phosphorus free synthesis, and also correlation between accumulation of poly(A)$^+$RNA and alkaline protease synthesis in these bacteria [55]. Electrophoretic and immunological analyses of products obtained after translation of bacterial poly(A)$^+$RNA in oocytes of the clawed frog (*Xenopus*) allowed identification of certain hydrolases [56]. Thus, it was shown, that the extracellular hydrolytic enzyme mRNA of *B. intermedius* was polyadenylated and this increased its stability, which promoted maintenance of high level of hydrolase syntheses during attenuation and full stop of the growth of the culture.

Our data suggest differences in localization and secretion of these proteins. In contrast to RNase which bacteria almost completely released into the cultivation medium (>99%) [57], the phosphatase (70%) and protease (~10%) were found in catalytically active form in the cytoplasmic membrane. Active RNase was found in the cell wall; it represented less than 1% of the secreted enzyme [58]. Immunoblotting did not reveal the presence of RNase or its intermediate products in the membrane fraction [59]. In contrast to RNase, secretion of protease and phosphatase occurs via two stage mechanism which involves formation of active membrane bound form [60, 61]. The terminal stage of secretion, release of phosphatase and protease, requires the presence of metal ions (Co^{2+} and Ca^{2+}). Metal cations do not influence RNase

export. The mutant protein with substitutions of amino acid residues involved formation of elements of secondary structure of the mature molecule was employed in the studies. Substitution of Trp34 for Asn involved in the formation of α-helical structure at the N-terminus of ribonuclease molecule caused blockade of protein translocation but did not prevent protein interaction with membrane [59]. It was concluded that effectiveness of RNase secretion depends on the rate of formation of secondary elements in the protein molecule. The RNase molecule is characterized by spontaneous formation of active conformation [62] which is realized after translocation and processing. Our data suggest that structural properties influence routes of their release from bacterial cells into the medium. Thus, later stages of exoprotein secretion in bacilli may be realized via different mechanisms that are determined by structural features of the secreted proteins.

We found hydrolases, particularly proteases, which bacteria secrete at later stages of spore formation, corresponding to spore maturation and sporangium autolysis [63]. Study of *B. intermedius* subtilisin gene expression by the recombinant strain of *B. subtilis* deficient in extracellular proteases revealed the same course of enzyme accumulation as in initial strain. Subtilisin was secreted into the medium at the initiation stage of spore formation in the culture, whereas maximal level of subtilisin secretion corresponded to late stages of sporulation. However, we observed some changes in physicochemical characteristics of the enzyme (e.g., insignificant shift of pH optimum for this protease). It is possible that changes in microenvironment may influence the process of enzyme activation. The process of spore formation is characterized by significant changes in the structure of the cell wall [64]. This leads to changes in composition and concentration of folding effectors (molecular chaperones involved in subtilisin maturation, Ca^{2+} concentration), which can influence formation of mature structure of this enzyme.

ROLE OF MATURE SEQUENCE IN SECRETION

Signal peptides of protein precursors in bacilli are replaceable and heterologic proteins are secreted by *B. subtilis* cells with different efficacy [65]. This suggests that mature sequence of exported polypeptides plays a certain role in protein secretion [66].

The kinetics of signal peptide cleavage was analyzed for a set of heterologic proteins of recombinant strains of *B. subtilis* [66, 67]. Hybrid proteins included sequences of each of four polypeptides, *E. coli* alkaline phosphatase, staphylococcus protein A (42 kD), RNase (EC 3.1.4.23, 12.382 kD), and *B. amyloliquefaciens* levansaccharase (EC 2.4.1.1, 50 kD), and one of signal peptides of the following extracellular proteins of *B. amyloliquefaciens*: alkaline protease (EC 3.4.21.62, 27.5 kD), neutral protease (EC 3.4.24.3, 38 kD), RNase, and levansaccharase. It was shown that in any of heterologic precursors the rate of signal peptide cleavage was determined by both signal peptide structure and the mature sequence [68]. Effects of most point mutations in the signal peptide of alkaline protease depended on the sequence of the mature protein (alkaline protease or alkaline phosphatase of *E. coli*) [66].

Deletion in the mature sequence of *B. licheniformis* subtilisin caused accumulation of protein precursor in the membrane [69]. It was concluded that secretion was damaged at the stage of protein translocation or protein release from the membrane. Certain evidence exists that the mature sequence influences protein release from the membrane after signal peptide removal: mutant *B. subtilis* levansaccharase with substitution at the 336 position was not released from the membrane [70].

ROLE OF CELL WALL IN LATE STAGES OF SECRETION

Cell wall is the other critical factor at final stages of protein secretion in bacilli. Cell wall forms the microenvironment for completion of protein export and formation of mature conformation of exported proteins. In bacilli cell wall consists of peptidoglycan with covalently bound teichoic acids. In this microenvironment protein binding *in vivo* is realized together with changes occurring in this compartment during translocation. In vegetative bacillus cells the cell wall and teichoic acids contain D-alanine residues, which finally determine density of total negative charge of the cell surface [71]. Free hydroxyl groups in teichoic acids may be substituted for D-alanine residues; this reduces negative charge of the cell wall and its ability to bind cations required as cofactors for post-translational protein folding. Experiments with label revealed that effectiveness of exoprotein secretion by protoplasts which are formed after cell wall removal was significantly lower than by intact *B. subtilis* cells [72]. This demonstrates cell wall requirement for effective secretion. Proteins translocated through the cell wall in partially or completely folded state may be subjected to temporal association with cell wall components. In the case of α-amylase secretion longer time interval required for its release into the medium than in the case of levansaccharase may be attributed to their different affinity to cell wall components [47].

Impairments in exoprotein secretion in mutants of *B. subtilis* deficient by foldase PrsA3 were overcome by suppressor mutation in the dlt-operon, controlling D-alanylation of teichoic acids [73]. In wild type *B. subtilis* cells free hydroxyl groups of phosphoric acid residues in lipoteichoic and teichoic acids may be substituted with D-alanine residues by 50 and 25%, respectively [71]. *dlt*-Mutants were characterized by the absence of D-alanine

in teichoic acids of the cell wall. The change in total charge of cell wall promoted activation of some proteins localized on the outer surface of the membrane; such activation of PrsA-lipoprotein resulted in stimulation of release of polypeptides which are folded in PrsA-dependent manner [73]. Increased total negative charge of cell wall increases affinity with respect to Ca^{2+} and this promotes posttranslational folding of Ca^{2+}-dependent proteins. Reduction of PrsA level below 200 molecules per cell abolished cell growth due to impairments of cell wall polymer synthesis. *dlt*-Mutation not only suppressed *prsA3*-mutation but also maintained cell growth under PrsA deficit. Thus, the absence of D-alanylation stabilizes proteins essential for synthesis of cell wall components.

For characterization of the interaction between a secreted exoprotein and cell wall components, refolding kinetics of levansaccharase and α-amylase of *B. subtilis* was investigated *in vitro* in the presence of polyphosphates of various length ($2 < n < 65$) at pH 7 and 37°C [74]. Soluble anionic polymers simulated the role of teichoic acids. In contrast to α-amylase, levansaccharase rapidly formed native structure in the presence of polyphosphates ($n > 16$) even in the absence of Ca^{2+}. This effect explains the different rate of translocation of these proteins through the cell wall.

Study of expression of the α-amylase gene under control of the sacR signaling sequence in strain *B. subtilis* degU32(Hy) by using impulse protein labeling revealed that the rate of release of enzyme molecules after cleavage of signal peptide decreased in the period of steady state phase ($t_{1/2} = 2$ min versus $t_{1/2} = 3.2$ min in the exponential phase of growth) [75]. Effectors controlling the stage of release (PrsA level or Ca^{2+}-binding properties of cell wall) remained unchanged. The difference in effectiveness of secretion might be attributed to changes in structural components of the cell wall, which are observed at different stages of growth of microorganisms. It is also possible that stability of α-amylase mRNA varies at different phases of growth [9].

Thus, folding properties play essential role in the mechanism of protein secretion in bacilli. Folding kinetics of secreted proteins depends on composition and amount of specific effectors in microenvironment and also on chemical composition and total charge of the cell surface, which are determined by structural components of the cell wall. Realization of certain mechanisms of protein release into the medium depends on properties of the protein structure.

SECRETION AND SPORULATION

Studies of protein secretion are especially interesting at the steady state growth phase which is accompanied by initiation of spore formation. The formation of spore-specific proteins is characterized by diversity of posttranslational modifications, including proteolytic processing, protein-dependent transfer directed to correct interaction of individual components, and delivery into spore compartments [76]. Endospores are covered by a protein shell, which provides resistance of spores and their ability to monitor environment and rapid onset of germination under favorable conditions [77]. The spore shell consists of a group of heterogeneous proteins, which includes more than 20 polypeptides of molecular masses ranged from 6 to 69 kD. They form three structural layers. Assembly of proteins constituting this shell is a rather slow process that is accompanied by significant morphological changes in the cell [77, 78]. A protein of molecular mass of 30 kD was isolated from spores of *B. subtilis gerE*-mutant; this protein is crucial for assembly of the spore shell [76]. The N-terminal sequence of this protein shared homology with product of gene *tasA*. Expression of this gene is controlled by σH and formation of mature conformation of TasA depends on SipW signal peptidase type I [76]. The mature polypeptide was found in supernatant of sporulating culture and also inside cells right after the beginning of sporulation. So, it was concluded that TasA is secreted into the prespore compartment at the stage of septa formation. Later, after prespore engulfing by the parent cell TasA is activated in the septoproximal field located between two spore membranes; this is required to center the organization of internal layers of forming spore shell. The protein TasA represents the first example of polypeptide involvement in assembly of proteins of the spore shell. The production of these proteins is not specific for the parent cell [76, 79, 80]. Based on these data, the authors consider protein secretion as a specific mechanism required for spore shell formation. It is possible that assembly of this shell is initiated during septa formation via synthesis of certain proteins that are directly secreted to the prespore compartment. This is consistent with results that synthesis of components of secretion machinery (Sec-proteins) is required at intermediate stages of differentiation [81]. Spontaneous suppressor mutations obtained on the basis of secA12-mutant of *B. subtilis* (deficient by SecA) are localized in transmembrane SecY-protein. The expression of early sporulation genes *kinA* and *spo0A* encoding histidine kinase and transcription factor for induction of sporulation genes was preserved in the suppressor mutants [81]. It was concluded that translocase functioning coupled to Sec-proteins is necessary for the development of sporulation in *B. subtilis* cells.

ROLE OF FtsH-PROTEIN IN SECRETION IN BACILLI

FtsH-protein belongs to the prokaryotic AAA-protein family (ATPases associated with multiple cell activi-

ty). The AAA-proteins are involved in secretion of polypeptides, assembly of integral membrane proteins, proteolysis, cell cycle control, etc. FtsH is an integral protein of 70.7 kD. Its N-terminus contains hydrophobic segments which are anchored at phospholipid membranes by two transmembrane loops [82]. The region including from 25 to 95 amino acid residues flanks at the outer side of the membrane. The C-terminal conservative domain is exposed to the cytoplasm; it contains ATP-binding domain and a sequence similar to active site of Zn-dependent metalloproteases [83]. There are about 400 molecules of FtsH-protein in the cell. Mutation analysis revealed that FtsH is involved in proteolytic degradation of SecY molecules unbound to SecE; FtsH may also influence protein secretion processes [84].

In *E. coli* FtsH-protein is a membrane-bound ATP-dependent metalloprotease acting with respect to transcription factor σ^{32} [83]. This suggests the existence of a new mechanism of gene regulation involving rapid control of gene activity by hydrolysis of key regulatory proteins by cell proteases [85-87].

A gene encoding FtsH was identified in *B. subtilis* cells [88]. Mutations in *B. subtilis ftsH* gene were accompanied by impaired cell growth and assembly of membrane penicillin-binding proteins, PBP2A, PBP2B, PBP4 [89]. PBP2A-protein is involved in synthesis of *B. subtilis* cell wall [90]. The content of this protein in membrane progressively decreases during transition to sporulation when there is no need for cell wall elongation. PBP2B-protein is employed for asymmetrical septa formation and therefore it is required for cell growth and sporulation [91]. The function of PBP4 remains unclear. In *B. subtilis* FtsH is involved in regulation of PBP protein assembly.

In bacilli *ftsH*-mutation causes cell response after spore formation and action of high salt concentrations [89]. Cell functioning in normal medium and renewal of cell growth are accompanied by 2-fold increase of *ftsH* transcription. Increase of *ftsH* gene expression during stress in bacilli resembles response of *E. coli* cell to heat shock [83]. Since FtsH is anchored to the membrane by two transmembrane domains, this site might function as a part of sensor domain responding to stress conditions. Structural features of FtsH are suggested to be similar with integral membrane components of signal transducing systems, but search for homology of genes encoding corresponding proteins did not give positive results [82].

Certain evidence exists that *B. subtilis* ecs-operon encodes ABC-transporter, which is a part of a signal-transducing mechanism; it is also involved in regulation of synthesis of components of protein secretion machinery and synthesis of exoproteins [92].

It was shown that *ftsH* gene expression reduced during cell differentiation; *spoIIE* gene was not expressed in *ftsH*-mutant [89]. SpoVM-polypeptide which consists of 26 amino acid residues (3 kD) inhibited activity of FtsH-protease [93, 94]. Production of σ-factors (σ^E and σ^K) and spore-specific genes was impaired in *spoVM*- and *ftsH*-mutants. Analysis of double mutations *spoVM-ftsH* revealed, that the interaction between SpoVM and FtsH is required for termination of engulfment of prespore by the parent cell. At a new level of complexity the transcription control by means of peptide inhibitors circulating between inner and outer cell surfaces as information messengers plays a key role for substantiation of a new regulatory mechanism in microbial systems [49, 95, 96].

There is increasing evidence that FtsH has multiple functions in prokaryotic cells. Mutations of the *ftsH* gene are highly pleiotropic; they affect cell growth, viability, events associated with protein–membrane interaction, secretion, assembly of polypeptides in membrane, and spore formation. Mutation analysis revealed that FtsH may function as chaperone promoting protein secretion and proteolysis of various proteins including transcription control factors.

Thus, after transmembrane translocation secreted proteins pass through the cell wall and undergo posttranslational modifications at the final stage of secretion. This results in formation of stable three-dimensional structures of protein molecules, which are preserved over the whole period of their life in the surrounding medium. The rate and effectiveness of polypeptide folding are critical factors in production of corresponding proteins and enzymes. Maintenance of posttranslational control on the structure and function of protein depends on the presence of various folding effectors, molecular chaperones, and also ATP-dependent proteases, which can recognize specific domains, exposed at unfolded sites of the exported polypeptides [97]. Molecular chaperones and folding effectors localized at the external surface of bacilli are involved in protein folding and prevention of protein aggregation, whereas energy-dependent proteases irreversible eliminate damaged proteins. This discrimination of functions of effectors, chaperones, and proteases determines fate of secreted protein (whether or not the secreted protein will be cleaved before termination of formation of its mature conformation) [97]. Studies on the role of cell wall in protein secretion in bacilli suggest that it is an active participant of this process; the cell wall represents the microenvironment for later stages of exoprotein secretion and not just a mechanical permeability barrier as suggested earlier [98].

The author is deeply grateful to M. A. Nesmeyanova for her generous help in the formation of concept for this paper and to I. B. Leshchinskaya and G. N. Rudenskaya who made significantly contribution to formation of scientific ideology of the author of this paper.

This work was supported by grants from the Russian Foundation for Basic Research (No. 01-04-48037), CRDF (Rec 007), and St. Petersburg University.

REFERENCES

1. Nesmeyanova, M. A. (1985) *Problems of Biochemistry and Physiology of Microorganisms* [in Russian], Pushchino, pp. 159-167.
2. Nesmeyanova, M. A. (1986) *Usp. Sovr. Biol.*, **102**, 179-192.
3. Nesmeyanova, M. A. (1990) *Advances in Science and Technology. Biotechnology* [in Russian], Vol. 22, VINITI, Moscow.
4. Pugsley, A. P. (1993) *Microbiol. Rev.*, **57**, 50-108.
5. Simonen, M., and Palva, I. (1993) *Microbiol. Rev.*, **57**, 109-137.
6. Priest, F. G. (1985) *Microbiol. Sci.*, **2**, 278-282.
7. Kontinen, V. P., and Sarvas, M. (1993) *Mol. Microbiol.*, **8**, 727-737.
8. Kunst, F., Ogasawara, N., Moszer, I., Albertini, A. M., Alloni, G., Azevedo, V., Bertero, M. G., Bessieres, P., Bolotin, A., Borchert, S., Borriss, R., Boursier, L., Brans, A., Braun, M., Brignell, S. C., Bron, S., Brouillet, S., Bruschi, C. V., Caldwell, B., Capuano, V., Carter, N. M., Choi, S. K., Codani, J. J., Connerton, I. F., Danchin, A., et al. (1997) *Nature*, **390**, 249-256.
9. Pereira, Y., Chambert, R., Leloup, L., Daguer, J. P., and Petit-Glatron, M. F. (2001) *Microbiology*, **147**, 1331-1341.
10. Bolhuis, A., Tjalsma, H., Smith, H. E., de Jong, A., Meima, R., Venema, G., Bron, S., and van Dijl, J. M. (1999) *Appl. Environ. Microbiol.*, **65**, 2934-2941.
11. Wu, S. C., Ye, R., Wu, X. C., Ng, S. C., and Wong, S. L. (1998) *J. Bacteriol.*, **180**, 2830-2835.
12. Bolhuis, A., Sorokin, A., Azevedo, V., Ehrlich, S. D., Braun, P. G., de Jong, A., Venema, G., Bron, S., and van Dijl, J. M. (1996) *Mol. Microbiol.*, **22**, 605-618.
13. Saunders, C. W., Schmidt, B. J., Mallonee, R. L., and Guyer, M. S. (1987) *J. Bacteriol.*, **169**, 2917-2925.
14. Meens, J., Herbort, M., Klein, M., and Freudl, R. (1997) *Appl. Environ. Microbiol.*, **63**, 2814-2820.
15. Stephenson, K., and Harwood, C. R. (1998) *Appl. Environ. Microbiol.*, **64**, 2875-2881.
16. Driessen, A. J. M., Fekkes, P., and van der Wolk, J. P. W. (1998) *Curr. Opin. Microbiol.*, **1**, 216-222.
17. Bolhuis, A., Broekhuizen, C. P., Sorokin, A., van Roosmalen, M. L., Venema, G., Bron, S., Quax, W. J., and van Dijl, J. M. (1998) *J. Biol. Chem.*, **273**, 21217-21224.
18. Meyer, T. H., Menetret, J. F., Breitling, R., Miller, K. R., Akey, C. W., and Rapoport, T. A. (1999) *J. Mol. Biol.*, **258**, 1789-1800.
19. Swaving, J., van Wely, K. H., and Driessen, A. J. (1999) *J. Bacteriol.*, **181**, 7021-7027.
20. Economou, A. (1998) *Mol. Microbiol.*, **27**, 511-518.
21. Mori, H., Araki, M., Hikita, C., Tagaya, M., and Mizushima, S. (1997) *Biochim. Biophys. Acta*, **1326**, 23-36.
22. Leloup, L., Driessen, A. J., Chambert, R., and Petit-Glatron, M. F. (1999) *J. Bacteriol.*, **181**, 1820-1826.
23. Knonova, S. V., Zolov, S. N., Krupyanko, V. I., and Nesmeyanova, M. A. (2000) *Biochemistry (Moscow)*, **65**, 1075-1081.
24. Tjalsma, H., Noback, M. A., Bron, S., Venema, G., Yamane, K., and van Dijl, J. M. (1997) *J. Biol. Chem.*, **272**, 25983-25992.
25. Tjalsma, H., Kontinen, V. P., Pragai, Z., Wu, H., Meima, R., Venema, G., Bron, S., Sarvas, M., and van Dijl, J. M. (1999) *J. Biol. Chem.*, **274**, 1698-1707.
26. Tjalsma, H., Bolhuis, A., Jongbloed, J. D. H., Bron, S., and van Dijl, J. M. (2000) *Microbiol. Mol. Biol. Rev.*, **64**, 515-547.
27. Tjalsma, H., Stover, A. G., Driks, A., Venema, G., Bron, S., and van Dijl, J. M. (2000) *J. Biol. Chem.*, **275**, 25102-25108.
28. Bolhuis, A., Matzen, A., Hyyrylainen, H. L., Kontinen, V. P., Meima, R., Chapuis, J., Venema, G., Bron, S., Freudl, R., and van Dijl, J. M. (1999) *J. Biol. Chem.*, **274**, 24585-24592.
29. Leloup, L., le Saux, J., Petit-Glatron, M. F., and Chambert, R. (1999) *Microbiology*, **145**, 613-619.
30. Leskela, S., Wahlstrom, E., Kontinen, V. P., and Sarvas, M. (1999) *Mol. Microbiol.*, **31**, 1075-1085.
31. Rudd, K. E., Sofia, H. J., Koonin, E. V., Plunkett, G. I., Lazar, S., and Rouviere, P. E. (1995) *Trends Biochem. Sci.*, **20**, 12-14.
32. Jacobs, M., Andersen, J. B., Kontinen, V., and Sarvas, M. (1993) *Mol. Microbiol.*, **8**, 957-966.
33. Vitikainen, M., Pummi, T., Airaksinen, U., Wahlstrom, E., Wu, H., Sarvas, M., and Kontinen, V. P. (2001) *J. Bacteriol.*, **183**, 1881-1890.
34. Beveridge, T. J. (1995) *ASM News*, **61**, 125-130.
35. Doyle, R. J. (1990) in *Metal Ions and Bacteria* (Beveridge, T. J., and Doyle, R. J., eds.) John Wiley, New York, pp. 275-293.
36. Chambert, R., and Petit-Glatron, M. F. (1990) *FEBS Lett.*, **275**, 61-64.
37. Simon, S. M., Peskin, C. S., and Oster, G. F. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 3770-3774.
38. Pantoliano, M. W., Whitlow, M., Wood, J. F., Rollence, M. L., Finzel, B. C., Giliand, G. L., Poulos, T. L., and Bryan, P. N. (1988) *Biochemistry*, **27**, 8311-8317.
39. Hughes, A. H., Hancock, I. C., and Baddiley, J. (1973) *Biochem. J.*, **132**, 83-93.
40. Petit-Glatron, M.-F., Grajcar, L., Munz, A., and Chambert, R. (1993) *Mol. Microbiol.*, **9**, 1097-1106.
41. Petit-Glatron, M. F., Monteil, I., Benyahia, F., and Chambert, R. (1990) *Mol. Microbiol.*, **4**, 2063-2070.
42. Petit-Glatron, M.-F., Benyahia, F., and Chambert, R. (1987) *Eur. J. Biochem.*, **163**, 379-387.
43. Shioi, J. I., Matsura, S., and Imae, Y. (1980) *J. Bacteriol.*, **144**, 891-897.
44. Kemper, M. A., Urrutia, T. J., Beveridge, A. L., and Doyle, R. J. (1993) *J. Bacteriol.*, **175**, 5690-5696.
45. Chambert, R., Haddaoui, E. A., and Petit-Glatron, M. F. (1995) *Microbiology*, **141**, 997-1005.
46. Leloup, L., Haddaoui, E. A., Chambert, R., and Petit-Glatron, M.-F. (1997) *Microbiology*, **143**, 3295-3303.
47. Haddaoui, E. A., Leloup, L., Petit-Glatron, M. F., and Chambert, R. (1997) *Eur. J. Biochem.*, **249**, 505-509.
48. Stephenson, K., Carter, N. M., Harwood, C. R., Petit-Glatron, M. F., and Chambert, R. (1998) *FEBS Lett.*, **430**, 385-389.
49. Stephenson, K., Jensen, C. L., Jorgensen, S. T., Lakey, J. H., and Harwood, C. R. (2000) *Biochem J.*, **350**, 31-39.
50. Raivio, T. L., and Silhavy, T. J. (1999) *Curr. Opin. Microbiol.*, **2**, 159-165.
51. Stover, A. G., and Driks, A. (1999) *J. Bacteriol.*, **181**, 7065-7069.
52. Grishina, I. B., Bolotina, I. A., Esipova, N. G., Pavlovsky, A. G., and Makarov, A. A. (1989) *Mol. Biol. (Moscow)*, **23**, 1455-1468.

53. Itskovich, E. L., Balaban, N. P., Mardanova, A. M., Shakirov, E. V., Sharipova, M. R., Leshchinskaya, I. B., Ksenofontov, A. L., and Rudenskaya, G. N. (1987) *Biokhimiya*, **62**, 60-65.
54. Sharipova, M. R., Balaban, N. P., Nekhotyaeva, N. V., Mardanova, A. M., Dementiev, A. A., and Leshchinskaya, I. B. (1996) *Biochem. Mol. Biol. Int.*, **38**, 753-761.
55. Romakhina, E. R., Bagaeva, T. V., Sharipova, M. R., Kozyreva, E. A., and Leshchinskaya, I. B. (1988) *Mikrobiologiya*, **57**, 421-425.
56. Sharipova, M. R., Romakhina, E. R., Bagaeva, T. V., Balaban, N. P., and Leshchinskaya, I. B. (1989) *Mikrobiologiya*, **58**, 365-369.
57. Sharipova, M. V., Vershinina, V. I., Balaban, N. P., and Leshchinskaya, I. B. (1987) *Mikrobiologiya*, **56**, 805-811.
58. Sharipova, M. R., Filatova, S. V., Vinter, V. G., and Leshchinskaya, I. B. (1984) *Mikrobiologiya*, **53**, 563-567.
59. Sharipova, M. R. (2000) *Hydrolases from Bacillus intermedius: Purification*, *Characterization and Localization*: Doctoral dissertation [in Russian], Kazan State University, Kazan.
60. Sharipova, M. R., Balaban, N. P., and Leshchinskaya, I. B. (2000) *Mikrobiologiya*, **69**, 197-202.
61. Sharipova, M. R., Shakirov, E. V., Balaban, N. P., Gabdrakhmanova, L. A., Shilova, M. A., Rudenskaya, G. N., and Leshchinskaya, I. B. (2000) *Mikrobiologiya*, **69**, 660-667.
62. Hartley, R. W. (1989) *Trends Biochem. Sci.*, **14**, 450-454.
63. Sharipova, M. R., Balaban, N. P., Gabdrakhmanova, L. A., Shilova, M. A., Kadyrova, Yu. M., Rudenskaya, G. N., and Leshchinskaya, I. B. (2002) *Mikrobiologiya*, **71**, 494-499.
64. Errington, J. (1993) *Microbiol. Rev.*, **57**, 1-33.
65. Sarvas, M. (1988) *Curr. Top. Microbiol. Immunol.*, **125**, 103-126.
66. Nagarajan, V. (1993) in *Bacillus subtilis and Other Gram-Positive Bacteria: Biochemistry*, *Physiology*, *and Molecular Biology* (Sonenshein, A. L., Hoch, J. A., and Losick, R., eds.) American Society for Microbiology, Washington, pp. 713-726.
67. Nagarajan, V. (1990) *Meth. Enzymol.*, **185**, 214-223.
68. Chen, M., and Nagarajan, V. (1993) *Mol. Gen. Genet.*, **239**, 409-415.
69. Schulein, R., Kreft, J., Gonski, S., and Goebel, W. (1991) *Mol. Gen. Genet.*, **227**, 137-143.
70. Benyahia, F., Chambert, R., and Petit-Glatron, M. F. (1988) *J. Gen. Microbiol.*, **134**, 3259-3268.
71. Perego, M., Glaser, P., Minutello, A., Strauch, M. A., Leopold, K., and Fischer, W. (1995) *J. Biol. Chem.*, **270**, 15598-15606.
72. Graham, L. L., and Beveridge, T. J. (1994) *J. Bacteriol.*, **176**, 1413-1421.
73. Hyyrylainen, H. L., Vitikainen, M., Thwaite, J., Wu, H., Sarvas, M., Harwood, C. R., Kontinen, V. P., and Stephenson, K. D. (2000) *J. Biol. Chem.*, **275**, 26696-26703.
74. Chambert, R., and Petit-Glatron, M. F. (1999) *FEMS Microbiol Lett.*, **179**, 43-47.
75. Haddaoui, E., Chambert, R., Petit-Glatron, M. F., Lindy, O., and Sarvas, M. (1999) *FEMS Microbiol Lett.*, **173**, 127-131.
76. Serrano, M., Zilhao, R., Ricca, E., Ozin, A. J., Moran, C. P., and Henriques, A. O. (1999) *J. Bacteriol.*, **181**, 3632-3643.
77. Henriques, A. O., and Moran, C. P. (2000) *Methods*, **20**, 95-110.
78. Stragier, P., and Losick, R. (1996) *Annu. Rev. Genet.*, **30**, 297-341.
79. Stover, A. G., and Driks, A. (1999) *J. Bacteriol.*, **181**, 5476-5481.
80. Stover, A. G., and Driks, A. (1999) *J. Bacteriol.*, **181**, 1664-1672.
81. Kobayashi, H., Ohashi, Y., Nanamiya, H., Asai, K., and Kawamura, F. (2000) *FEMS Microbiol. Lett.*, **184**, 285-289.
82. Tomoyasu, T., Yamanaka, K., Murata, K., Suzaki, T., Boulos, P., Kato, A., Niki, H., Hiraga, S., and Ogura, T. (1993) *J. Bacteriol.*, **175**, 1352-1357.
83. Tomoyasu, T., Gamer, J., Bukau, B., Kanemori, M., Mori, H., Rutman, A. J., Oppenheim, A., Yura, T., Yamanaka, K., Niki, H., Hiraga, S., and Ogura, T. (1995) *EMBO J.*, **14**, 2551-2560.
84. Kihara, A., Akiyama, Y., and Ito, K. (1995) *Proc. Natl. Acad. Sci. USA*, **92**, 4532-4536.
85. Gottesman, S. (1999) *Curr. Opin. Microbiol.*, **2**, 142-147.
86. Wu, W. F., Zhou, Y., and Gottesman, S. (1999) *J. Bacteriol.*, **181**, 3681-3687.
87. Rudner, D. Z., Fawcett, P., and Losick, R. (1999) *Proc. Natl. Acad. Sci. USA*, **96**, 14765-14770.
88. Ogasawa, N., Nakai, S., and Yoshikawa, H. (1994) *DNA Res.*, **1**, 1-14.
89. Lysenko, E., Ogura, T., and Cutting, S. M. (1997) *Microbiology*, **143**, 971-978.
90. Sowell, M. O., and Buchanan, C. E. (1983) *J. Bacteriol.*, **153**, 1331-1337.
91. Yanouri, A., Daniel, R. A., Errington, J., and Buchanan, C. E. (1993) *J. Bacteriol.*, **175**, 7604-7616.
92. Leskela, S., Wahlstrom, E., Hyyrylainen, H. L., Jacobs, M., Palva, A., Sarvas, M., and Kontinen, V. P. (1999) *Mol. Microbiol.*, **31**, 533-543.
93. Cutting, S., Anderson, M., Lysenko, E., Page, A., Tomoyasu, T., Tatematsu, K., Tatsuta, T., Kroos, L., and Ogura, T. (1997) *J. Bacteriol.*, **79**, 5534-5542.
94. Prajapati, R. S., Ogura, T., and Cutting, S. M. (2000) *Biochim. Biophys. Acta*, **1475**, 353-359.
95. Hoch, J. A. (1998) *Curr. Opin. Microbiol.*, **1**, 170-174.
96. Hoch, J. A. (2000) *Curr. Opin. Microbiol.*, **3**, 165-170.
97. Wickner, S., Maurizi, M. R., and Gottesman, S. (1999) *Science*, **286**, 1888-1893.
98. Archibald, A. R., Hancock, I. C., and Harwood, C. R. (1993) in *Bacillus subtilis and Other Gram-Positive Bacteria: Biochemistry*, *Physiology*, *and Molecular Biology* (Sonenshein, A. L., Hoch, J. A., and Losick, R., eds.) American Society for Microbiology, Washington, pp. 381-410.