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***Appl. Environ. Microbiol.* 2002, 68(1):227. DOI: 10.1128/AEM.68.1.227-234.2002.**

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 2002, p. 227–234 Vol. 68, No. 1
0099-2240/02/$04.00+0 DOI: 10.1128/AEM.68.1.227–234.2002

Optimization of the Cell Wall Microenvironment Allows Increased Production of Recombinant *Bacillus anthracis* Protective Antigen from *B. subtilis*

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Received 11 July 2001/Accepted 8 October 2001

The stability of heterologous proteins secreted by gram-positive bacteria is greatly influenced by the microenvironment on the *trans* side of the cytoplasmic membrane, and secreted heterologous proteins are susceptible to rapid degradation by host cell proteases. In *Bacillus subtilis,* degradation occurs either as the proteins emerge from the presecretory translocase and prior to folding into their native conformation or after the native conformation has been reached. The former process generally involves membrane- and/or cell wall-bound proteases, while the latter involves proteases that are released into the culture medium. The identification and manipulation of factors that influence the folding of heterologous proteins has the potential to improve the yield of secreted heterologous proteins. Recombinant anthrax protective antigen (rPA) has been used as a model secreted heterologous protein because it is sensitive to proteolytic degradation both before and after folding into its native conformation. This paper describes the influence of the microenvironment on the *trans* side of the cytoplasmic membrane on the stability of rPA. Specifically, we have determined the influence of net cell wall charge and its modulation by the extent to which the anionic polymer teichoic acid is D-alanylated on the secretion and stability of rPA. The potential role of the *dlt* operon, responsible for D-alanylation, was investigated using a *Bacillus subtilis* strain encoding an inducible *dlt* operon. We show that, in the absence of D-alanylation, the yield of secreted rPA is increased 2.5-fold. The function of D-alanylation and the use of rPA as a model protein are evaluated with respect to the optimization of *B. subtilis* for the secretion of heterologous proteins.

Bacillus anthracis, a gram-positive spore-forming bacterium, is the causative agent of anthrax. Expression of full virulence is determined by the production of two major virulence factors, the anthrax toxins and a poly-D-glutamic acid capsule, encoded by plasmids pXO1 and pXO2, respectively (14, 29). The products of three genes combine to form the two bipartite toxins of *B. anthracis*. Protective antigen (PA) and edema factor (EF) combine to form edema toxin, while PA combines with lethal factor (LF) to form lethal toxin.

It has long been known that an extracellular protective antigen is produced when *B. anthracis* is grown in vivo (6). PA alone is nontoxic and induces a protective immune response (45). The currently licensed vaccines against anthrax in both the United States and United Kingdom are based on precipitated cell extracts of *B. anthracis* (United States,V770-NP1-R absorbed onto aluminum hydroxide gel; United Kingdom, Sterne $34F_2$ precipitated by alum) (15). Although the Sterne United Kingdom vaccine strain is attenuated, it is still categorized as an Advisory Committee on Dangerous Pathogens (ACDP) category III pathogen; therefore, vaccine production requires the use of expensive high-level containment facilities. Besides PA, these vaccines contain trace amounts of EF and LF (the other potentially toxic components) and a variety of medium-derived proteins, any of which may contribute to the short-lived side effects recorded in some individuals following vaccination (47).

To overcome these problems, attempts have been made to produce recombinant protective antigen (rPA) in a variety of heterologous hosts, including *B. subtilis* (20), *Escherichia coli* (39), *Salmonella enterica* serovar Typhimurium (12), *Lactobacillus casei* (54), and viral hosts, including baculovirus and vaccinia virus (19). Of the hosts tested so far, only *B. subtilis* has been found to give yields of rPA that are compatible with large-scale production (3, 4, 5).

Bacillus species in general, and *B. subtilis* in particular, are highly valued commercially for their ability to secrete proteins

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directly into the culture supernatant at gram-per-liter concentrations (13). The high secretion capacity, coupled with the ease of fermentation and impressive safety record, has identified *B. subtilis* as an important host for efficient large-scale secretion of a wide variety of heterologous proteins.

Secretion-competent preproteins are actively transported to and across the cytoplasmic membrane by the translocase complex, utilizing energy from ATP and the proton motive force (40, 46). Following translocation, signal peptidases process the preprotein, mediating the cleavage of the N-terminal signal peptide and the release of the unfolded mature protein on the *trans* side of the membrane. Many heterologous proteins are subject to rapid degradation by host cell-associated proteases before they are able to fold into their mature, usually more proteolytically resistant, fully folded conformation (20, 27, 43). Consequently, the speed and efficiency of in vivo folding of secreted proteins directly affect their overall yield.

B. subtilis secretes seven extracellular proteases into the culture medium. Native secretory proteins which have coevolved alongside these proteases are, for the most part, resistant to their proteolytic activities. In contrast, heterologous proteins are often highly susceptible to these proteases, prompting attempts to engineer multiply-protease-deficient strains of *B. subtilis*, such as WB600 (53). However, despite the fact that six extracellular protease genes have been inactivated in this strain, so that it exhibits just 0.32% of the proteolytic activity of the wild type, the proteolytic degradation of heterologous proteins has not been eliminated (28, 42). Even proteins that are highly resistant to proteases in their fully folded state can be rapidly and substantially degraded following translocation, suggesting the existence of a degradation pathway associated with the cell wall. This activity has been attributed, in part at least, to the product of the *wprA* gene, which encodes a cell wall-associated serine protease (26). Consequently, a *B. subtilis* strain that was not able to express its *wprA* gene exhibited twice the α-amylase activity of the wild-type strain (43).

The identification of posttranslocational factors that participate in protein folding may facilitate the optimization of heterologous protein secretion. Factors known to influence the folding of secreted proteins include extracellular folding factors (e.g., PrsA) and the physicochemistry of the cell wall. PrsA is an extracytoplasmic molecular chaperone located on the *trans* side of the cytoplasmic membrane which functions as a peptidyl-prolyl-*cis*-*trans*-isomerase (PPI). PrsA catalyzes the conversion of proline residues from the *cis* configuration into the *trans* configuration. This is the rate-limiting step for the folding of many secretory proteins (50). Overproduction of PrsA has been shown to increase the production of α-amylase, proteases, and single-chain antibody fragments (23, 50, 52).

The efficient folding of a number of secretory proteins is dependent on the presence of divalent metal cations Fe^{3+} or Ca^{2+} (10, 25, 34, 44). The importance of Ca^{2+} to rPA stability has been shown by growing *B. subtilis* in minimal medium with and without $CaCl_2$ (10 mg/liter); in the absence of Ca^{2+}, little if any rPA was detected by Western blotting (Leslie Baillie, personal communication). The availability of metal cations to act as folding effectors is determined by their concentration in the environment and the properties of the anionic polymers (teichoic and teichuronic acids) covalently attached to peptidoglycan in the cell wall.

Teichoic acids, including wall teichoic acids (WTA) and lipoteichoic acids (LTA), are essential components of the cation exchange system between the cell envelope and the surrounding environment. They confer a high density of negative charge on the cell wall and the capacity to attract metal cations such as Ca^{2+}, Fe^{3+}, and Mg^{2+} (7, 17, 34). The ion exchange activities of teichoic acids are mediated by the presence of ester-linked D-alanyl residues that control the overall negative charge of the cell wall (16). The free amino moiety of each D-alanyl residue is protonated at physiological pH, effectively neutralizing the negative charge of a neighboring phosphate residue on the teichoic acid molecule. Significantly, when extracellular cation concentrations are high, the effects of D-alanylation are marginal, whereas at low cation concentrations cell wall binding is significantly increased in the absence of alanylation (24).

The D-alanylation of LTA and WTA in *B. subtilis* is determined by the products of the *dlt* operon. This operon is composed of five genes, *dltA* to *dltE*. Insertional inactivation of genes *dltA* to *dltD* results in the complete inhibition of D-alanylation (32) but does not affect cell growth or metabolism. However, these mutants are marginally more vulnerable to cell lysis (51). Recent work has shown that inactivation of *dltB* or *dltD* markedly increases the stability of certain secretory proteins, especially those prone to proteolysis (18).

As a model secretory protein, *B. anthracis* protective antigen has advantages over other model proteins, such as α-amylases, in that it is subject to proteolysis at both cell-associated and extracellular locations. In this paper, we show that the degree of cell wall alanylation influences the secretion of rPA by *B. subtilis*. In particular, we show the kinetics of rPA secretion and the relative contributions of proteolytic events that occur before and after this protein is released from the cell. The analysis and optimization of rPA secretion are likely to provide insights into the key features of the *B. subtilis* secretion pathway, which could be adapted to improve the yield of secreted heterologous proteins.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids are listed in Table 1. All strains were maintained on antibiotic medium 3 plates (Difco Laboratories Ltd., East Molesley, United Kingdom) or in liquid culture in 2xYT broth (1.6% tryptone, 1% yeast extract, 0.5% NaCl) buffered with 0.2 M MES (morpholineethanesulfonic acid, pH 6.0). Pulse-chase labeling experiments were carried out in Spizizen's minimal salts medium (SMS) (41). For plasmid maintenance, growth medium contained ampicillin (100 μg/ml), erythromycin (1 μg/ml), kanamycin (5 μg/ml), and lincomycin (25 μg/ml), as necessary.

Stability of rPA and AmyL in spent culture medium. Cultures of *B. subtilis* 168 and *B. subtilis* WB600 were grown for 24 h in 20 ml of 2xYT to allow the full expression of extracellular proteases. The cells were removed by centrifugation (10,000 × *g*, 30 min, 4°C), and the spent medium containing extracellular proteases was filtered through 0.45-μm-pore Acrodisc-32 filters (Pall Gelman Sciences, Ann Arbor, Mich.). Aliquots (5 μl of 1 mg/ml) of purified rPA (31) and AmyL (42) were added to 300 μl of culture supernatant and incubated at 37°C. Samples (20 μl) were removed at intervals over a 36-h period to determine rPA and AmyL levels by Western blotting.

DNA manipulations and PCR. Plasmid DNA extraction, restriction endonuclease digestion, ligation, agarose gel electrophoresis, and transformation of electrocompetent *E. coli* cells were carried out as described previously (36). Enzymes, molecular size markers, and deoxynucleotides were purchased from Roche Diagnostics Ltd. (Lewes, United Kingdom) or Amersham Pharmacia Biotech Ltd. (Little Chalfont, United Kingdom). Extraction of *B. subtilis* DNA and transformation of *B. subtilis* by the Groningen method were done according to Bron (9). The PCR was carried out as described previously (37) with *Pfu*

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Comments	Reference or source
Strains			
B. subtilis 168	*trpC2*	Wild-type *B. subtilis*	1
B. subtilis DN1885	*amyE amyR2*	α-Amylase-negative derivative of *B. subtilis* 168	Novo Nordisk A/S
B. subtilis WB600	*trpC2* Δ*nprE* Δ*aprE* Δ*epr* Δ*bpf* Δ*mpr* Δ*nprB*	*B. subtilis* with six extracellular protease genes knocked out	53
B. subtilis JET1	*amyE*, pPA101, Km^r	DN1885 with rPA plasmid pPA101	This study
B. subtilis JET2	*amyE*, pPA101, IPTG-inducible *dltA-E*; Km^r Em^r Lm^r	DN1885 JET1 with pMUTin4*dltA*FP integrated at *dltA*	This study
E. coli XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′-*proAB lacI*^{q}*Z M15*ΔTn*10* (Tct^r)]		Stratagene
Plasmids			
pPA101	rPA; Km^r	Derivative of pUB110 containing recombinant *pagA*	20
pMUTin4	Amp^r Em^r Lm^r *spoVG-lacZ* P_{spac}		48
pMUTin4*dltA*FP	Km^r Amp^r Em^r Lm^r	Integrated into the *B. subtilis* chromosome at *dltA*	This study

(Stratagene Europe, Amsterdam, The Netherlands) or *Taq* DNA polymerase (Promega UK Ltd., Southampton, United Kingdom), using the following cycling program: 1 cycle of 5 min at 94°C and then 35 cycles of 1 min at 94°C, 1 min at 60°C, and 1 to 4 min at 72°C, depending on the size of the PCR product (36).

Plasmid pJET1 was created by amplification of the 5′ end of the *dltA* gene by PCR, using primers DLT-F (5′-CGC**GGATCC**GGAGAGAGAATAACTATG AAAC-3′) and DLT-R (5′-CGC**GGATCC**TTCGTTTTCCAGCAGTTCTTCC-3′). These primers were designed to include *Bam*HI restriction sites (shown in bold) to facilitate cloning. The recombinant plasmids were transformed into competent *B. subtilis* cells, and transformants were selected on antibiotic medium 3 plates containing erythromycin and lincomycin. The mutants were analyzed by PCR to confirm the integration of a single copy of the plasmid into the target gene on the chromosome, using a strategy similar to that described previously (37).

SDS-PAGE and Western blotting. Cultures were grown in 20 ml of 2xYT broth buffered with 0.2 M MES (pH 6.0) at 37°C. Samples of culture supernatant (20 μl), taken hourly throughout growth, were mixed with 20 μl of 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and then boiled for 5 min. The samples were run on a 10% polyacrylamide gel alongside prestained low-range molecular size markers (Bio-Rad, Hercules, Calif.). Proteins were transferred from the gel onto a 0.4-μm nitrocellulose membrane (Anderman Ltd., Kingston upon Thames, United Kingdom). rPA protein was detected using rabbit polyclonal anti-PA antiserum followed by anti-rabbit immunoglobulin-horseradish peroxidase conjugate (Dako Immunochemicals A/S, Glostrup, Denmark). Protein bands were visualized by immersion in freshly prepared Western blot staining solution containing 4-chloro-1-naphthol and hydrogen peroxide.

Analysis of D-alanine esters of wall teichoic acids. Cultures were grown in 20 ml of 2xYT broth buffered with 0.2 M MES (pH 6.0) at 37°C. Culture samples (1 ml) were removed at hourly intervals and centrifuged, and the resulting pellet was stored at −20°C. Cell disruption, alkaline hydrolysis of D-alanine from WTA, and colorimetric assays to determine liberated D-alanine concentrations were performed as described previously (18).

Pulse-chase experiments. Bacterial cultures were grown to exponential phase in SMS, indicated by an optical density at 660 nm of 1.0. The culture was pulse-labeled with L-[^{35}S]methionine (561 kBq/μl; Amersham International Plc.), as described previously (43, 49). Following the chase, pairs of samples (750 μl) were taken at various times (0, 2, 5, 7, 10, 15, 30, 45, and 60 min). The first sample, containing cells and growth medium, represented total protein, while the second sample, obtained by filtration through a 0.45-μm-pore polyvinylidene difluoride filter (Whatman, Maidstone, United Kingdom), represented protein secreted into the culture medium. rPA in the samples was immunoprecipitated with rPA-specific antibodies, and the immune complexes were recovered by centrifugation. These were subjected to SDS-PAGE and analyzed by fluorography. The relative amounts of radiolabeled rPA at each time point were determined by phosphoimaging (PhosphorImager; Molecular Dynamics) and analyzed using ImageQuant software (version 3.22; Molecular Dynamics).

β-Galactosidase activity. Cultures were grown in 20 ml of 2xYT broth at 37°C. Samples were removed to determine optical density at 660 nm and β-galactosidase activity as described previously (30).

Surface charge topology mapping. A three-dimensional model of the surface topology of rPA was determined using the published crystallographic protein structure data of anthrax protective antigen (35) (Swiss-Prot accession number P13423). Loop regions for which the structure could not be determined were omitted, as well as the two bound Ca^{2+} ions. A three-dimensional solvent-accessible contour map of rPA was created using the Sybyl 6.7 program (Tripos Ltd., St. Louis, Mo.) using the Connolly surface method (11). The spatial distribution of electrostatic potential was determined using the Poisson-Boltzmann equation.

RESULTS

Stability of rPA in spent culture medium. The onset of stationary phase triggers the synthesis by *B. subtilis* of a number of extracellular proteases (33). We therefore determined the effects of extracellular proteases released into the culture medium on the stability of rPA. Stability was determined in spent culture medium used for the growth of the wild-type *B. subtilis* 168 and derivative WB600, which is deficient in six extracellular proteases (53). A protease-resistant heterologous protein,

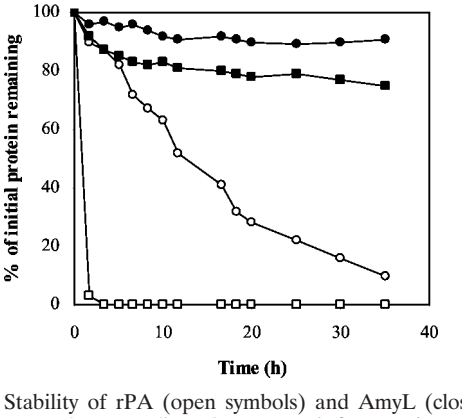

FIG. 1. Stability of rPA (open symbols) and AmyL (closed symbols) in spent culture medium from *B. subtilis* 168 (squares) and WB600 (circles). The proteins were detected by Western blotting and are expressed as a percentage of the initial protein concentration.

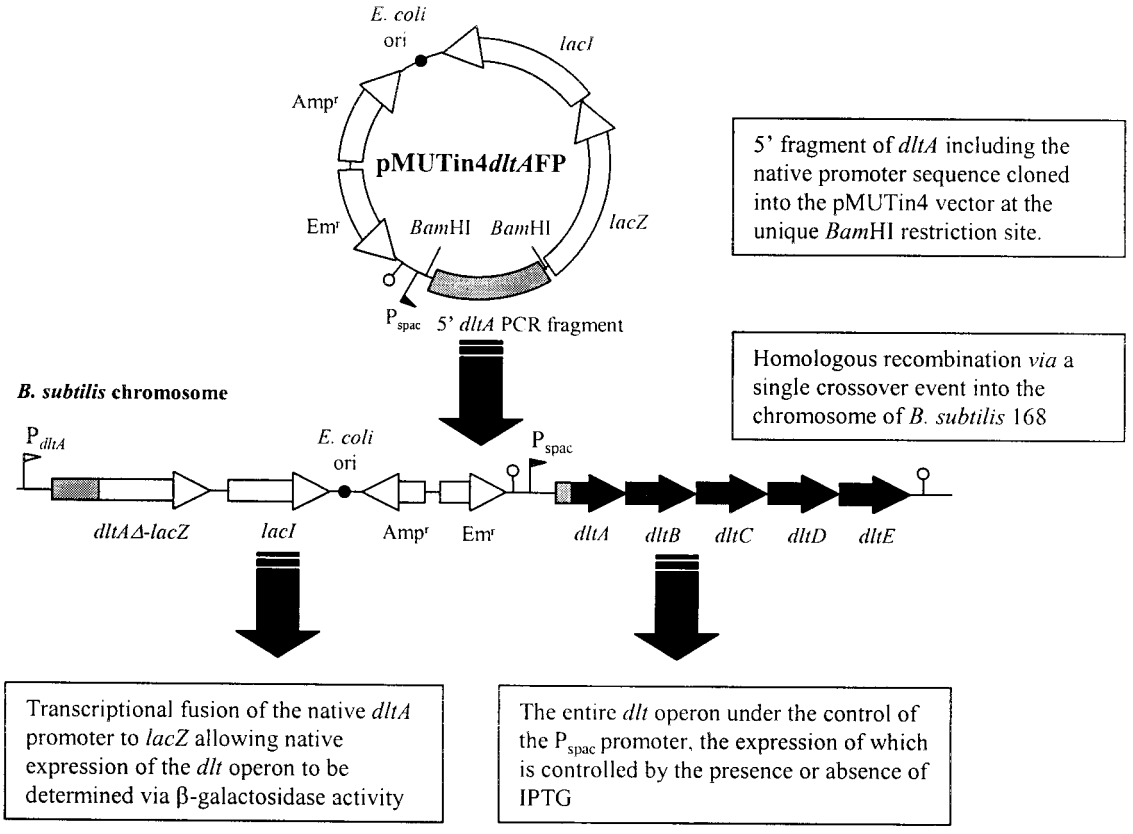

FIG. 2. Construction of an IPTG-inducible *dlt* operon in *B. subtilis* DN1885 by cloning a 5′ fragment of the *dltA* gene into the *B. subtilis* integration vector, pMUTin4. The open flag represents the native *dlt* promoter, P_{dlt}, while the IPTG-inducible P_{spac} promoter is depicted by a solid flag.

Bacillus licheniformis α-amylase (AmyL), was included for comparison (43).

In the presence of spent culture medium from the wild type, rPA had a half-life of approximately 50 min (Fig. 1). In spent medium from the protease-deficient strain WB600, the half-life was extended to 12 h. The rapid proteolysis in the *B. subtilis* 168 supernatant is typical of a wide variety of heterologous proteins secreted by *B. subtilis*. AmyL, however, was markedly more stable; in the presence of wild-type levels of proteases, 80% of the initial protein was still present after 35 h, and this was increased to 90% in spent medium from the protease-deficient strain.

Construction of a mutant with an inducible *dlt* operon. To determine the effects of D-alanylation on the secretion and stability of rPA, we constructed a strain of *B. subtilis* in which the entire *dlt* operon was placed under the control of an isopropylthio-β-D-galactopyranoside (IPTG)-inducible P_{spac} promoter (Fig. 2). A 419-bp fragment from the 5′ end of the *dltA* gene was amplified from *B. subtilis* 168 chromosomal DNA using oligonucleotide primers DLT-F and DLT-R. The product was digested with *Bam*HI, cloned in the pMUTin4 integrational vector (48), and used to transform *E. coli* XL1-Blue (Stratagene, La Jolla, Calif.). Diagnostic PCR, using vector- and insert-specific primers, was used to screen for the presence and correct orientation of the insert in the recombinant plasmids, and their structure was confirmed by sequencing the entire insert and vector sequences on either side of the cloning site (data not shown).

The resulting plasmid, pMUTin4*dltA*FP, was used to transform *B. subtilis* JET1 to generate *B. subtilis* JET2 *dltA*::pMUTin4. The recombinant strain was tested by PCR to verify the integrity of the construct. In this strain the expression of the *dlt* operon was under the control of the P_{spac} promoter, allowing the expression of the operon to be induced by the addition of IPTG to the growth medium (48).

Influence of the *dlt* operon on WTA D-alanylation and rPA secretion. *B. subtilis* 168 and JET2 *dltA*::pMUTin4 were grown at 37°C in 2xYT, the latter with or without IPTG, until the onset of stationary phase. Samples were taken to monitor growth, the extent of WTA D-alanylation, and the yield of secreted rPA. All of the strains exhibited similar growth kinetics (Fig. 3A), confirming that the *dlt* operon has no obvious effects on cell growth (32).

The D-alanylation of WTA was measured to determine the activity of the *dlt* operon (Fig. 3B). In wild-type *B. subtilis,* the extent of D-alanylation increased during transition to stationary phase and then decreased during stationary phase. If the pH of the culture medium is increased to 8.1, the ester bond linking the D-alanyl residue to teichoic acid becomes labile (22) and as a result, no D-alanylated WTA could be detected (data not shown). Analysis of the non-IPTG-induced JET2 *dltA*::pMUTin4 showed, as expected, an absence of D-alanine

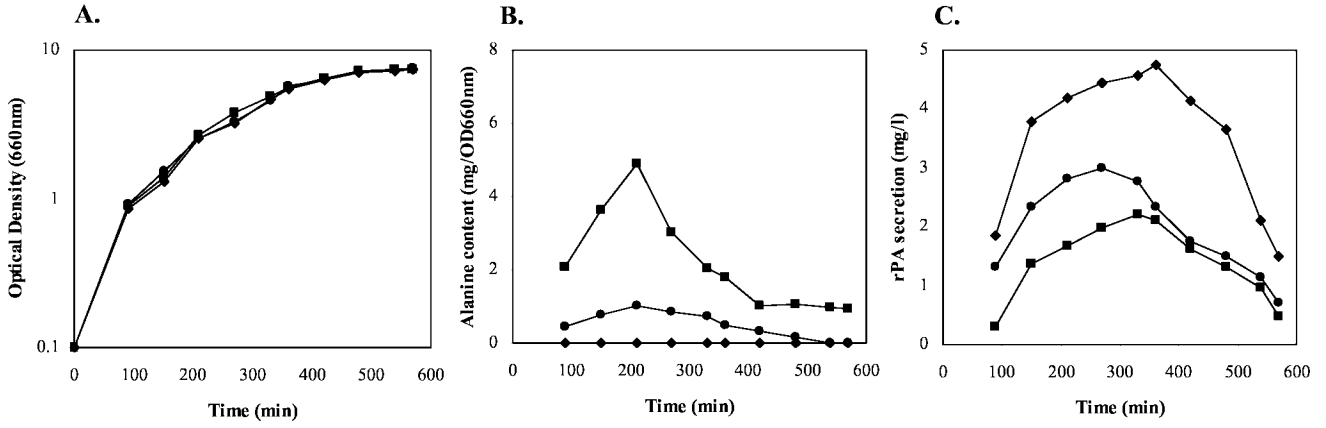

FIG. 3. Phenotypic effects of *dlt* operon modulation using *B. subtilis* 168 (■) and *B. subtilis* JET2 *dltA*::pMUTin4 with (●) and without (♦) 1 mM IPTG. (A) Growth kinetics in 2xYT–0.2 M MES (pH 6.5) determined at OD_{660}. (B) D-Alanyl ester content of isolated wall teichoic acids, detected by a colorimetric reaction. (C) Extracellular secretion of mature rPA into the culture medium, detected via Western blotting.

residues in WTA. In contrast, alanylation was partially restored when 1 mM IPTG was added to the culture medium. However, the level of alanylation in the induced mutant (≈1 mg of alanine/unit of culture OD) was only about one fifth of that observed in the wild type. Increasing the concentration of IPTG to 10 mM did not restore the level of alanylation to that of the wild type (data not shown). The failure to restore the level of alanylation in the JET2 *dltA*::pMUTin4 to that of the wild type probably reflects the fact that the operator in this construct has been optimized to achieve maximum repression under noninducing conditions, with consequential limitations to the extent to which the P_{spac} promoter can be induced (48).

The secretion of rPA was determined by Western blotting (Fig. 3C). Wild-type *B. subtilis* secreted rPA to a maximum concentration of 2 mg/liter, while that of the noninduced *B. subtilis* JET2 *dltA*::pMUTin4 was 4.75 mg/liter. Induction of the *dlt* operon in the mutant with 1 mM IPTG resulted in the synthesis of an intermediate concentration of rPA (3 mg/liter), which was compatible with the degree of alanylation observed in this strain.

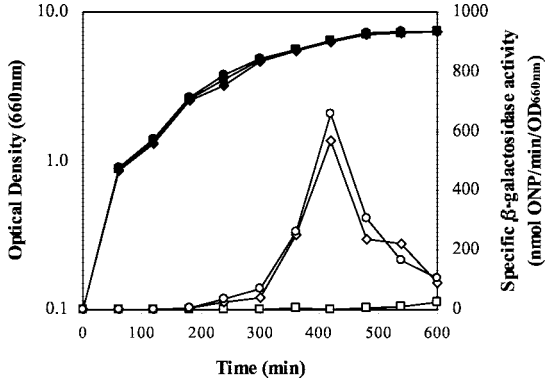

FIG. 4. β-Galactosidase assay for transcriptional activity of the *dlt* operon using a *dltA*::lacZ transcriptional fusion. Growth (solid symbols) and β-galactosidase activity (open symbols) were measured in cultures of *B. subtilis* 168 (squares) and *B. subtilis* JET2 *dltA*::pMUTin4 with (diamonds) and without (circles) 1 mM IPTG.

Transcriptional activity of the *dlt* operon. The activity of the native *dlt* promoter was analyzed in *B. subtilis* JET2 *dltA*::pMUTin4, in which the native promoter was transcriptionally fused to a *lacZ* reporter gene, enabling the transcriptional activity to be monitored via β-galactosidase activity (Fig. 4). Transcription of the *dlt* operon remained low during exponential growth but, in agreement with previous reports (32), was transiently induced during transition into stationary phase. These data are consistent with the observed levels of WTA alanylation (Fig. 3B). The transcription of the native *dlt* promoter was independent of the presence of IPTG, indicating that this operon is not autoregulated by products of the *dlt* operon.

Pulse-chase analysis of rPA processing. The kinetics of rPA secretion were determined by coupled pulse-chase/immunoprecipitation experiments. Wild-type *B. subtilis* and JET2 *dltA*::pMUTin4, with and without 1 mM IPTG, were grown in Spizizen's minimal medium to exponential phase (OD_{660} of approximately 1) and pulse-chased with L-[^{35}S]methionine. Radioactively labeled rPA was immunoprecipitated, separated by SDS-PAGE, and visualized by autoradiography (Fig. 5A). The precursor form of rPA (mature protein plus signal peptide), with a molecular mass of 85.8 kDa, was clearly distinguishable from that of the mature form (82.7 kDa).

In the wild type, the processing of rPA from precursor to mature form (Fig 5A) was relatively slow compared to other secreted *Bacillus* proteins (e.g., AmyL) (43). Following the addition of the nonradioactive chase (T_0), only 50% of the radioactively labeled rPA was in the mature form. The maximal amount of labeled mature rPA was recorded approximately 5 min following the chase, with the labeled precursor form disappearing after approximately 10 min. The amount of mature rPA decreased rapidly; at 30 min postchase, half had been degraded, and virtually no mature rPA was detected after 60 min (Fig. 5A). The secreted mature rPA, representing 20% of the maximum synthesized during the pulse, reached a peak at 7 min and was completely degraded after approximately 40 min. These data confirm that rPA is subject to extensive degradation during or shortly after translocation through the cy-

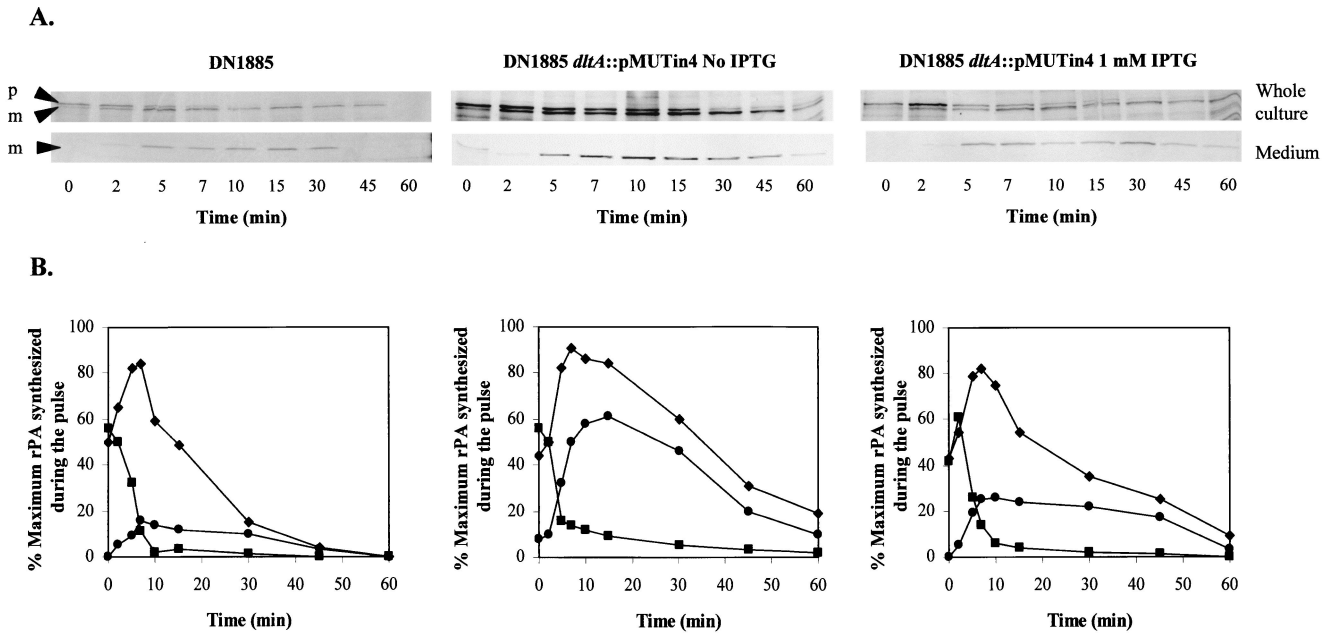

FIG. 5. Secretion of rPA from exponentially growing cultures of *B. subtilis* in the presence and absence of the products of the *dlt* operon. Representative data are shown for pulse-chase experiments carried out on strains *B. subtilis* 168 and *B. subtilis* JET2 *dltA*::pMUTin4 with and without 1 mM IPTG. (A) Autoradiographs of pulse-chased rPA following immunoprecipitation with anti-rPA polyclonal antibodies and SDS-PAGE. The top gels in panel A indicate precursor (p) and mature (m) rPA immunoprecipitated from whole-culture samples, and the bottom gels indicate mature rPA released into the culture medium. (B) Quantification by phosphoimaging of the different forms of rPA at different times following the chase. ■, rPA precursor; ♦, mature rPA in whole-culture samples; ●, mature rPA released into the growth medium. The amount of each form of rPA is expressed as a percentage of the total rPA (precursor plus mature) synthesized during the pulse.

toplasmic membrane and after secretion into the growth medium.

Processing of precursor rPA in *B. subtilis* JET2 *dltA*::pMUTin4 (Fig. 5B and 5C) appeared to be slower than that of the wild type, and significant amounts of the precursor form were detected 15 min after the pulse. In the absence of induction (i.e., no alanylation), the maximal level of labeled mature rPA was detected approximately 15 min after the chase and remained relatively stable for the next 30 min. Even at 60 min postchase, significant amounts of rPA were detectable (Fig. 5B), both in whole culture samples and secreted into the culture medium.

Induction of the *dlt* operon in *B. subtilis* JET2 *dltA*::pMUTin4 (Fig 5C) reduced the stability of rPA to a level that was intermediate between that of the wild-type and the noninduced mutant strain (Fig 5B). Processing of the precursor protein into mature protein occurred at an intermediate rate. Mature rPA peaked after 7 min at 50% of the total synthesized during the pulse (cf. *B. subtilis* 168, 15%, and *B. subtilis* JET2 *dltA*::pMUTin4 without IPTG, 60%). Similarly, the secretion of rPA into the culture medium peaked at 30% of the maximum at 7 min, and it was degraded relatively slowly thereafter. In both the wild type and the mutant, there was a consistent difference between the amount of mature rPA detected in the whole culture and that secreted into the culture medium. This difference is likely to be accounted for by rPA trapped in the matrix of the cell wall.

Characterization of the surface topology of rPA. Protective antigen from *B. anthracis* has a calculated pI of 5.58. In order to determine the distribution of electrostatic potential on the surface of rPA, a three-dimensional solvent-accessible model was generated. The model shows clear variations in charge distribution; the surface is predominantly negatively charged, but with the strongly positively and negatively charged regions projecting from the main body of the protein. An exception is a concentration of negatively charged residues in domain 1 (35) and a strongly electropositive region corresponding to the convergence of three lysine residues.

The distribution of surface charge is likely to influence the interaction of rPA with the negatively charged cell wall of *B. subtilis* during secretion. The negatively charged face of rPA is likely to be repelled by the cell wall, whereas the positively charged projections may facilitate cell wall binding (44). The latter may be responsible for the discrepancy between the amount of rPA synthesized and that released into the culture medium (Fig. 5).

DISCUSSION

Obtaining high yields of secreted rPA from *B. subtilis* has been hampered by marked proteolytic degradation (5). Attempts to eliminate this degradation using *B. subtilis* WB600, a protease-deficient strain in which six out of seven extracellular protease genes have been inactivated (4, 53), have only been partially successful. Here we report a 15-fold increase in the half-life (from 0.8 to 12 h) of rPA in spent culture medium from a protease-deficient strain compared with the wild type. This was in marked contrast to *B. licheniformis* α-amylase,

which was stable over extended periods, even in spent culture medium derived from the wild type. rPA therefore represents a useful model for proteins that are subject to rapid proteolysis by *B. subtilis* extracellular proteases.

The recent observation that incompletely folded secretory proteins are more susceptible to proteolysis that their natively folded counterparts has focused attention on the identification of cotranslocational and posttranslocational events that influence the secretion of proteins from *B. subtilis* (8, 18, 43). Proteins may be most susceptible to proteolysis as they emerge from the translocase, and factors that increase the rate at which they achieve their native state are likely to promote protein stability and hence increase the overall recoverable yields. Thus, characterization of this environment and factors involved in efficient protein folding may provide insights for the optimization of heterologous proteins.

The microenvironment on the *trans* side of the cytoplasmic membrane into which proteins are secreted is controlled and maintained by the physicochemical properties of the membrane and the cell wall. The net charge in this region is modulated by the extent of D-alanylation of wall and lipoteichoic acids, which in turn affect the concentration of metal cations such as Ca^{2+}, Fe^{2+}, and Mg^{2+} at the cell membrane/wall microenvironment (7, 17, 18). These cations are required as folding factors for a variety of secreted proteins, including α-amylase and levansucrase (10, 25, 34, 44). The presence of calcium ions in the native structure of anthrax protective antigen means that metal ions are also likely to be important for the folding of this protein (35). In the absence of D-alanylation, the negative charge density in the wall increases, as does its affinity for metal cations (24). This in turn increases the availability of these cations to act as folding effectors and hence increases the rate of protein folding (18).

In contrast to previous studies in which the *dltB* and *dltD* genes have been insertionally inactivated (18), we have constructed a mutant in which the expression of the *dlt* operon has been placed under the control of the IPTG-inducible P_{spac} promoter (48). In comparison to the wild type, the yield of secreted rPA, as detected by Western blotting, was significantly increased in the noninduced mutant.

Analysis of rPA secretion kinetics by pulse-chase showed that rPA was stabilized in the absence of D-alanylation, leading to the release of increased amounts of mature rPA secreted into the culture medium. However, there was a consistent difference between the total amount of rPA synthesized during the pulse and the amounts released into the culture medium. This difference probably represents cell-associated degradation by wall- and/or membrane-associated proteases occurring shortly after translocation of the protein across the cytoplasmic membrane. In addition, the rate of precursor processing and cell-associated degradation is higher in the wild type than in the nonalanylated mutant.

The pulse-chase experiments also highlight a discrepancy, of approximately 10%, between the amount of mature rPA in the whole culture and that secreted into the culture medium. This may represent an interaction between a portion of the rPA protein and the cell wall. Physical-chemical interactions between the cell wall and secreted proteins have been suggested to inhibit the release of certain heterologous proteins (38). Although there is no definitive in vivo evidence for such interactions, except for native wall-associated proteins (2), studies using chimeric α-amylases with modified net charge have shown that positively charged proteins may be retarded by electrostatic interactions with the cell wall (21, 44). Analysis of the surface charge topology of rPA indicated that one face was negatively charged, while the opposing face had a number of positively charged projections that may facilitate interactions with the cell wall.

The use of *B. subtilis* as a host provides many advantages over the currently licensed method for the production of anthrax protective antigen. However, rPA, like many other heterologous proteins, is subjected to proteolytic attack by host cell proteases. The use of rPA as a model protein for investigating cell-associated and extracellular factors affecting protein stability is likely to prove valuable, not only for the production of the next generation of anthrax vaccines, but also for the secretion of other heterologous proteins of commercial and medical importance from *B. subtilis.*

ACKNOWLEDGMENTS

We thank S.-L. Wong for *B. subtilis* strain WB600, S. D. Ehrlich for pMUTin4, J. Miller for purified rPA, and B. Lingard for assistance with the computer modeling.

This work was supported by a BBSRC Case studentship, between the School of Biochemistry and Genetics, University of Newcastle, United Kingdom, and Dstl, Porton Down, Salisbury, United Kingdom.

REFERENCES

1. **Anagnostopouloulos, C., and J. Spizizen.** 1961. Requirements for transformation in *Bacillus subtilis*. J. Bacteriol. **81:**741–746.
2. **Archibald, A. R., I. Hancock, and C. Harwood.** 1993. Cell wall structure, synthesis and turnover, p. 381–410. *In* A. L. Sonenshein, J.A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other gram-positive bacteria. American Society for Microbiology, Washington, D.C.
3. **Baillie, L. W. J., M. Johnson, and R. J. Manchee.** 1994. Evaluation of *Bacillus subtilis* strain IS53 for the production of *Bacillus anthracis* protective antigen. Lett. Appl. Microbiol. **19:**225–227.
4. **Baillie, L. W. J., P. Moore, and R. J. Manchee.** 1996. Development of a *Bacillus subtilis* based system for the expression of the protective antigen of *Bacillus anthracis. In* Proceedings of the International Workshop on Anthrax, 19–21 September 1995, Winchester, United Kingdom. Salisbury Med. Bull. **87**(Spec. Suppl.)**:**133–135.
5. **Baillie, L., A. Moir, and R. Manchee.** 1998. The expression of the protective antigen of *Bacillus anthracis* in *Bacillus subtilis*. J. Appl. Microbiol. **84:**741–746.
6. **Belton, F. C., and R. E. Strange.** 1954. Studies on a protective antigen produced *in vitro* from *Bacillus anthracis*: medium and methods of purification. Br. J. Exp. Pathol. **35:**144–152.
7. **Beveridge, T. J., and R. G. Murray.** 1980. Sites of metal deposition in the cell wall of *Bacillus subtilis*. J. Bacteriol. **141:**876–887.
8. **Bolhuis, A., H. Tjalsma, K. Stephenson, C. R. Harwood, G. Venema, S. Bron, and J. M. van Dijl.** 1999. Different mechanisms for thermal inactivation of *Bacillus subtilis* signal peptidase. J. Biol. Chem. **274:**15865–15868.
9. **Bron, S.** 1990. Plasmids, p. 75–174. *In* C. R. Harwood and S. M. Cutting (ed.), Molecular biological methods for *Bacillus*. John Wiley & Sons Ltd., Chichester, United Kingdom.
10. **Chambert, R., F., E. A. Haddaoui, and M.-F. Petit Glatron.** 1995. *Bacillus subtilis* levansucrase: the efficiency of the second stage of secretion is modulated by external factors assisting folding. Microbiology **141:**997–1005.
11. **Connolly, M. L.** 1983. Solvent-accessible surfaces of proteins and nucleic acids. Science **221:**709–713.
12. **Coulson, N. M., M. Fulop, and R. W. Titball.** 1994. *Bacillus anthracis* protective antigen, expressed in *Salmonella typhimurium* SL 3261, affords protection against anthrax spore challenge. Vaccine **12:**1395–1401.
13. **Ferrari, E., A. S. Jarnigin, and B. F. Schmidt.** 1993. Commercial production of extracellular enzymes, p. 917–937. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other gram-positive bacteria. American Society for Microbiology, Washington, D.C.
14. **Green, B. D., L. Battisti, T. M. Koehler, C. B. Thorne, and B. E. Ivins.** 1985. Demonstration of a capsule plasmid in *Bacillus anthracis*. Infect. Immun. **49:**291–297.
15. **Hambleton, P., J. A. Carman, and J. Melling.** 1984. Anthrax: the disease in relation to vaccines. Vaccine **2:**125–132.

16. **Heptinstall, S., A. R. Archibald, and J. Baddiley.** 1970. Teichoic acids and membrane function in bacteria. Nature **225:**519–521.
17. **Hughes, A. H., I. C. Hancock, and J. Baddiley.** 1973. The function of teichoic acids in cation control in bacterial membranes. Biochem. J. **132:**83–93.
18. **Hyyryläinen, H.-L., M. Vitikainen, J. Thwaite, H. Wu, M. Sarvas, C. R. Harwood, V. P. Kontinen, and K. Stephenson.** 2000. D-alanine substitution of teichoic acids as a modulator of protein folding and stability at the cytoplasmic membrane/cell wall interface of *Bacillus subtilis*. J. Biol. Chem. **275:** 26696–26703.
19. **Iacono-Connors, L. C., S. L. Welkos, B. E. Ivins, and J. M. Dalrymple.** 1991. Protection against anthrax with recombinant virus-expressed protective antigen in experimental animal. Infect. Immun. **59:**1961–1965.
20. **Ivins, B. E., and S. L. Welkos.** 1986. Cloning and expression of the *Bacillus anthracis* protective antigen gene in *Bacillus subtilis*. Infect. Immun. **54:**537–542.
21. **Jensen, C., K. Stephenson, S. T. Jørgensen, and C. R. Harwood.** 2000. Cell-associated degradation affects the yield of secreted engineered and heterologous proteins in the *Bacillus subtilis* expression system. Microbiology **146:**2583–2594.
22. **Kiriukhin, M. Y., and F. C. Neuhaus.** 2001. D-alanylation of lipoteichoic acid: role of the D-alanyl carrier protein in acylation. J. Bacteriol. **183:**2051–2058.
23. **Kontinen, V. P., and M. Sarvas.** 1993. The PrsA lipoprotein is essential for protein secretion in *Bacillus subtilis* and sets a limit for high-level secretion. Mol. Microbiol. **8:**727–737.
24. **Lambert, P. A., I. C. Hancock, and J. Baddiley.** 1975. Influence of alanyl ester residues on the binding of magnesium ions to teichoic acids. Biochem. J. **151:**671–676.
25. **Leloup, L., E. A. Haddaoui, R. Chambert, and M.–F. Petit-Glatron.** 1997. Characterization of the rate-limiting step of the secretion of *Bacillus subtilis* α-amylase overproduced during the exponential phase of growth. Microbiology **143:**3295–3303.
26. **Margot, P., and D. Karamata.** 1996. The *wprA* gene of *Bacillus subtilis* 168, expressed during exponential growth, encodes a cell-wall-associated protease. Microbiology **142:**3437–3444.
27. **Meens, J., E. Frings, M. Klose, and R. Freudl.** 1993. An outer membrane protein (OmpA) of *Escherichia coli* can be translocated across the cytoplasmic membrane of *B. subtilis*. Mol. Microbiol. **9:**847–855.
28. **Meens, J., M. Herbort, M. Klein, and R. Freudl.** 1997. Use of the pre-pro part of the *Staphylococcus hyicus* lipase as carrier for the secretion of *Escherichia coli* outer membrane protein A (OmpA) prevents proteolytic degradation of OmpA by cell-associated protease(s) in two different gram-positive bacteria. Appl. Environ. Microbiol. **63:**2814–2820.
29. **Mikesell, P., B. E. Ivins, J. D. Ristroph, and T. M. Drier.** 1983. Evidence for plasmid-mediated toxin production in *Bacillus anthracis*. Infect. Immun. **39:** 371–376.
30. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **Miller, J., B. W. McBride, R. J. Manchee, P. Moore, and L. W. J. Baillie.** 1998. Production and purification of recombinant protective antigen and protective efficacy against *Bacillus anthracis*. Lett. Appl. Microbiol. **26:**56–60.
32. **Perego, M., P. Glaser, A. Minutello, M. A. Strauch, K. Leopold, and W. Fischer.** 1995. Incorporation of D-alanine into lipoteichoic acid and wall teichoic acid in *Bacillus subtilis*. J. Biol. Chem. **270:**15598–15606.
33. **Pero, J., and A. Sloma.** 1993. Proteases, p. 939–952. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other gram-positive bacteria. American Society for Microbiology, Washington, D.C.
34. **Petit-Glatron, M. F., L. Grajcar, A. Munz, and R. Chambert.** 1993. The contribution of the cell wall to a transmembrane calcium gradient could play a key role in *Bacillus subtilis* protein secretion. Mol. Microbiol. **9:**1097–1106.
35. **Petosa, C., R. J. Collier, K. R. Klimpel, S. H. Leppla, and R. C. Liddington.** 1997. Crystal structure of the anthrax toxin protective antigen. Nature **385:** 833–838.
36. **Prágai, Z., H. Tjalsma, A. Bolhuis, J. M. van Dijl, G. Venema, and S. Bron.** 1997. The signal peptidase II (*lsp*) gene of *Bacillus subtilis*. Microbiology **143:**1327–1333.
37. **Prágai, Z., and C. R. Harwood.** 2000. YsxC, a putative GTP-binding protein essential for the growth of *Bacillus subtilis* 168. J. Bacteriol. **182:**6819–6823.
38. **Saunders, C. W., B. J. Schmidt, R. L. Mallonee, and M. S. Guyer.** 1987. Secretion of human serum albumin from *Bacillus subtilis*. J. Bacteriol. **169:** 2917–2925.
39. **Sharma, M., P. K. Swain, A. P. Chopra, V. K. Chaudhary, and Y. Singh.** 1996. Expression and purification of anthrax toxin protective antigen from *Escherichia coli*. Protein Expr. Purif. **7:**33–38.
40. **Simonen, M., and I. Palva.** 1993. Protein secretion in *Bacillus* species. Microbiol. Rev. **57:**109–137.
41. **Spizizen, J.** 1958. Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleate. Proc. Natl. Acad. Sci. USA **44:**1072–1078.
42. **Stephenson, K.** 1996. Construction and use of chimeric α-amylases to study protein secretion in *Bacillus subtilis.* Ph.D. thesis. University of Newcastle upon Tyne, Newcastle upon Tyne, United Kingdom.
43. **Stephenson, K., and C. R. Harwood.** 1998. Influence of a cell-wall-associated protease on production of α-amylase by *Bacillus subtilis*. Appl. Environ. Microbiol. **64:**2875–2881.
44. **Stephenson, K., N. M. Carter, C. R. Harwood, M.-F. Petit-Glatron, and R. Chambert.** 1998. The influence of protein folding on the secretion of α-amylases from *Bacillus subtilis*. FEBS Lett. **430:**385–389.
45. **Thorne, C. B.** 1993. *Bacillus anthracis*, p. 113–124. *In* A. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other gram-positive bacteria. American Society for Microbiology, Washington, D.C.
46. **Tjalsma, H., A. Bolhuis, J. D. H. Jongbloed, S. Bron, and J. M. van Dijl.** 2000. Signal peptide-dependent protein transport in *Bacillus subtilis*: a genome-based survey of the secretome. Microbiol. Mol. Biol. Rev. **64:**515–547.
47. **Turnbull, P. C. B.** 2000. Current status of immunization against anthrax: old vaccines may be here to stay for a while. Curr. Opin. Infect. Dis. **13:**113–120.
48. **Vagner, V., E. Dervyn, and S. D. Ehrlich.** 1998. A vector for systematic gene inactivation in *Bacillus subtilis*. Microbiology **144:**3097–3104.
49. **van Dijl, J. M., A. de Jong, H. Smith, S. Bron, and G. Venema.** 1991. Signal peptidase I overproduction results in increased efficiencies of export and maturation of hybrid secretory proteins by *E. coli*. Mol. Gen. Genet. **227:** 40–48.
50. **Vitikainen, M., T. Pummi, U. Airaksinen, E. Wahlström, H. Wu, M. Sarvas, and V. P. Kontinen.** 2001. Quantitation of the capacity of the secretion apparatus and requirement for PrsA in growth and secretion of α-amylase in *Bacillus subtilis.* J. Bacteriol. **183:**1881–1890.
51. **Wecke, J., M. Perego, and M. Fischer.** 1996. D-alanine deprivation of *Bacillus subtilis* teichoic acids is without effect on cell growth and morphology but affects the autolytic activity. Microb. Drug Resist. **2:**123–129.
52. **Wu, S.-C., R. Ye, Y.-C. Wu, S.-C. Ng, and S.-L. Wong.** 1998. Enhanced secretory production of a single-chain antibody fragment from *Bacillus subtilis* by coproduction of molecular chaperones. J. Bacteriol. **180:**2830–2835.
53. **Wu, X., W. Lee, L. Tran, and S.-L. Wong.** 1991. Engineering a *Bacillus subtilis* expression-secretion system with a strain deficient in six extracellular proteases. J. Bacteriol. **173:**4952–4958.
54. **Zegers, N. D., E. Kluter, H. van Der Stap, E. van Dura, P. van Dalen, M. Shaw, and L. Baillie.** 1999. Expression of the protective antigen of *Bacillus anthracis* by *Lactobacillus casei*: towards the development of an oral vaccine against anthrax. J. Appl. Microbiol. **87:**309–314.