Production of *Bacillus anthracis* protective antigen is dependent on the extracellular chaperone, PrsA

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Running title: Extracytoplasmic chaperones of *B. anthracis*
[SUMMARY]

Protective antigen is a component of the *Bacillus anthracis* lethal and edema toxins, and the basis of the current anthrax vaccine. In its heptameric form, PA targets host cells and internalizes the enzymatically-active components of the toxins, namely lethal and edema factors. PA and other toxin components are secreted from *B. anthracis* using the Sec-dependent secretion pathway. This requires them to be translocated across the cytoplasmic membrane in an unfolded state and then to be folded into their native configurations on the trans side of the membrane, prior to their release from the environment of the cell wall. In this study we show that recombinant PA (rPA) requires the extracellular chaperone PrsA for efficient folding when produced in the heterologous host, *B. subtilis*; increasing the concentration of PrsA leads to an increase in rPA production. To determine the likelihood of PrsA being required for PA production in its native host, we have analyzed the *B. anthracis* genome sequence for the presence of genes encoding homologues of *B subtilis* PrsA. We identified three putative *B. anthracis* PrsA proteins (PrsAA, PrsAB and PrsAC) that are able to complement the activity of *B. subtilis* PrsA with respect to cell viability and rPA secretion, and as well as that of AmyQ, a protein previously shown to be PrsA-dependent.

[INTRODUCTION]

*Bacillus anthracis* is the etiological agent of anthrax – a historically well-documented disease. Anthrax affects mainly herbivorous animals such as sheep, cattle and wild herbivores, although all mammals are susceptible. The bacterium is transmitted predominately via spores rather than vegetative cells. Infection is usually acquired through the uptake of spores from soil or infected animal products by inhalation, ingestion or cutaneous abrasions. Fully virulent strains of *B. anthracis* possess two major virulence factors: the anthrax toxins and a $\gamma$-polyglutamic acid ($\gamma$-PGA) capsule.
The anthrax toxin proteins, edema factor (EF), lethal factor (LF) and protective antigen (PA) are encoded respectively by genes cya, lef and pag, located non-contiguously on plasmid pXO1 (185 kbp) (1). PA binds to either EF or LF to produce the binary edema (EdTx) or lethal (LeTx) toxins, respectively (5,6). Mice challenge experiments with isogenic strains of B. anthracis expressing either LeTx or EdTx indicate that, although these toxins act synergistically, LeTx is the key virulence factor (1).

The other main virulence factor, the γ-polyglutamic acid (γ-PGA) capsule, is encoded on plasmid pXO2 (95 kbp)(1). The capsule is only weakly immunogenic, and is therefore not suitable for vaccine purposes (2,3). γ-PGA is synthesized by enzymes encoded by the capA, capB and capC genes (4). The γ-PGA capsule forms the outermost element of the B. anthracis cell where it inhibits phagocytosis (2) by providing a monotonous linear polymer.

The current UK and USA human anthrax vaccines, although differing slightly, are based on PA (83 kDa.). The UK anthrax vaccine consists of an alum-precipitated culture filtrate from an aerobic static culture of B. anthracis strain Sterne 34F2, whilst the USA vaccine consists of an alhydrogel-adsorbed cell-free culture filtrate of B. anthracis V770-NP1-R, grown anaerobically in a fermenter. Both strains, although avirulent, must nevertheless be handled as class III pathogens. In an attempt to reduce the costs associated with handling strains of B. anthracis (5,6), various alternative production systems have been explored. However, with the notable exceptions of Bacillus subtilis and Escherichia coli, these have met with little success (7,8). Cloning pagA into B. subtilis strain IS53 (9) resulted in the secretion of recombinant PA (rPA) to a concentration of about 40 µg/ml; approximately three-fold higher than that obtained with B. anthracis Sterne (~15 µg/ml). Strain B. subtilis WB600, a multiply extracellular protease deficient strain (10,11), has been used to reduce degradation of rPA by co-produced proteases.
In an attempt to increase rPA production from *B. subtilis*, we have examined the role of PrsA on rPA secretion. PrsA is an essential lipoprotein component of the *B. subtilis* protein secretion pathway, where it functions on the *trans* side of the cytoplasmic membrane as a post-translocational folding factor (12). PrsA has been shown to be rate-limiting for the high level secretion of α-amylase. Increasing the cellular concentration of PrsA results in a corresponding increase in the amount of α-amylase secreted into the culture medium (13). Here we show that secretion of rPA, like that of α-amylase, is PrsA-dependent.

While the *B. subtilis* YacD, a PrsA paralogue, is not able to complement PrsA activity, we show here that three *B. anthracis* PrsA orthologues are able to do so, with respect to both viability and protein secretion in *B. subtilis*.

**EXPERIMENTAL PROCEDURES**

*Bacterial Strains, Plasmids and Growth Conditions* - Table I lists the bacterial strains and plasmids used. Strains were grown and maintained in Luria-Bertani (LB) medium (per liter: 10 g tryptone, 5 g yeast extract, 10 g NaCl), excepting for the determination of rPA production from strains MFJ683, MFJ943 and MFJ945, which were grown in complete anthracis (CA) medium (per liter: 35 g tryptone, 5 g yeast extract, 6 g Na$_2$PO$_4$.7H$_2$O, 1 g KH$_2$PO$_4$, 5.5 g NaCl, 40 mg L-tryptophan, 40 mg L-methionine, 5 mg thiamine, 25 mg uracil). Antibiotics were used at the following concentrations: ampicillin, 50 µg/ml; chloramphenicol, 5 µg/ml; erythromycin, 1 µg/ml; kanamycin, 10 µg/ml. Unless stated otherwise, isopropyl β-D thiogalactopyranoside (IPTG) was added at 1 mM and xylose at 1% (w/v) to induce gene expression from the P$_{spac}$ and P$_{xyz}$ promoters, respectively.

→ Table I
DNA Manipulation and Strain Construction - *B. subtilis* was transformed with plasmid DNA using the "Groningen" method (14). pRCW101 was constructed by amplifying a fragment (~300 bp) at the 5’-end of *prsA*, including ribosome binding site, from chromosomal DNA using oligonucleotide primers (*Bsu-prsA*-fwd 5’- CGCAAGCTTATTTGGAATGATTAGGAG –3’ and *Bsu-prsA*-rev 5’- CGCGGA TCCAGGGCAGTATATTGATCG-3’) with restriction endonuclease sites (*Bam*HI and *Hind*III) incorporated at their 5’-termini. The fragment was cloned into pMUTIN4 (15) and, following recovery in *E. coli*, pRCW101 was integrated into the *B. subtilis* chromosome, via a single crossover recombination at *prsA*. The resulting strain, RCW201, had a truncated non-functional version of *prsA* under the control of its native promoter and an intact copy of *prsA* under the control of the *Pspac* promoter.

Plasmids encoding *B. subtilis* (*Bsu*) and *B. anthracis* (*Ban*) *prsA* genes, pRCW207 (*Bsu-prsA*), pRCW208 (*Ban-prsAA*), pRCW209 (*Ban-prsAB*) and pRCW210 (*Ban-prsAC*), were constructed by amplifying the genes and associated ribosome binding sites from chromosomal DNA using oligonucleotide primers with restriction endonuclease sites (*Bam*HI and *Hind*III) incorporated at their 5’-termini (*Bsu-prsA* f-wd, 5’- CGCAAGCTTATTTGGAATGATTAGGAG -3’; *Bsu-prsA* rev2, 5’- CGCGGA TCCAGGGCAGTATATTGATCG-3’; *Ban-prsAA* f-wd3, 5’- GCCGAAGCTTGTAGGAGTGTATTCGAA-3’; *Ban-prsAA* rev4, 5’- GCCGAAGCTTGTAGGAGTGTATTCGAA-3’; *Ban-prsAB* f-wd3, 5’- GCCGAAGCTTATACATATTCCGAGTGAG-3’; *Ban-prsAB* rev4, 5’- GCCGAAGCTTATACATATTCCGAGTGAG-3’; *Ban-prsAC* f-wd5, 5’- GCCGAAGCTTATACATATTCCGAGTGAG-3’). The amplified fragments were cloned into pJPR1 using the multiple cloning site located immediately downstream of the *Pxy* promoter.
Adjacent to the cloned \textit{prsA} gene is a chloramphenicol resistance gene and this region of the plasmid is flanked by the 5’ and 3’ ends of \textit{B. subtilis amyE}. Strains in which plasmids pRCW207, pRCW208, RCW209 and pRCW210 had integrated into the chromosome of RCW201, \textit{via} a double crossover recombination at \textit{amyE} were isolated after selection of plates containing chloramphenicol.

\textit{Western blotting and dot-blot analyses for the quantitation of rPA} – For Western blotting, proteins separated by SDS-polyacrylamide gel electrophoresis were transferred to a nitrocellulose membrane (Protran, Schleicher & Schuell UK Ltd, London, UK) using a Mini-Trans-Blot system (BioRad, Hemel Hempsted, Herts, UK). For dot-blotting, sample of culture supernatant were transferred to a nitrocellulose membrane using a vacuum manifold (Invitrogen, Paisley, UK). Proteins were detected with in-house specific rabbit anti-PA antibodies and swine anti-rabbit IgG horse radish peroxidase conjugate (DakoCytomation Ltd, Ely, Cambs., UK) and visualised with the colorimetric developing agent 4-chloronapthol. The images generated by Western or dot blotting were analysed with QuantityOne (Bio-Rad Laboratories Ltd.; Hemel Hempstead, Herts., UK). Background values were removed prior to determining the intensities of individual protein bands. The relative band intensities were compared within and between lanes on a single gel.

\textit{Determination of \textalpha-amylase activity} - \textalpha-Amylase activity in culture supernatants was determined using the Phadebas Amylase Test (Pharmacia & Upjohn, Kalamazoo, MI, USA).

\textit{Multiple alignment of protein sequences} - The protein sequences were aligned using the ClustalW tool (http://www.ebi.ac.uk/clustalw). Calculation of the relative identity of the sequences was carried out using the Needleman-Wunsch global alignment algorithm, which is available within the EMBOSS suite of software at the Human Genome Project Resource Centre (http://www.hgmp.mrc.ac.uk).
RESULTS

Influence of PrsA on rPA secretion – Mutants of prsA encoding a defective PrsA protein exhibit a defect in the secretion of AmyQ, an α-amylase derived from B. amyloliquefaciens (12). The prsA3 mutation appears to affect PrsA folding, resulting in post-translocational proteolysis of PrsA and reduced PrsA activity (16). To determine whether PrsA influences the production of rPA, the pagA-encoding plasmid pYS5 (17) was introduced into B. subtilis strain MFJ945 in which the wild-type prsA gene was replaced with prsA3. The isogenic parental strain with a normal copy of prsA was MFJ943. MFJ945 (prsA3) and MFJ943 (prsA) were grown in CA medium and the cultures sampled during growth and stationary phases. Analysis of the culture supernatants by SDS-PAGE (Fig. 1) indicated that exponential and early stationary phase samples of MFJ943 contained a protein with the same relative mobility as rPA (lanes 2-3). This protein was absent from overnight cultures of this strain (lane 4). In contrast no rPA was detected in the supernatants of MFJ945 (prsA3) at any growth stage (lanes 5-7).

→ Fig. 1.

To confirm that PrsA influences the yield of rPA, strains were constructed so that the level of PrsA could be controlled. The pMUTIN4 integration vector (15) was used to construct B. subtilis strain RCW201 in which expression of prsA was under the control of the IPTG-inducible Pspac promoter. Since prsA is essential for viability (12), we confirmed that RCW201 had an absolute requirement for IPTG. At IPTG concentrations between 0.1 and 10mM, the growth rate and yield were similar to that of the wild-type strain, however, at lower concentrations, growth was characterized by a lag of several hours and the growth exclusively of IPTG-independent suppressor mutants. In contrast, the growth a strain in which the prsA homologue yacD was placed under Pspac control was not IPTG-dependent (data not shown).
RCW201PA (i.e. RCW201 with pPA101) was used to analyze the relationship between \textit{prsA} expression and the yield of secreted rPA. RCW201PA was grown in LB broth in the presence of various concentrations of IPTG (0.5-10 mM). rPA production was determined by quantitative Western blotting analysis of culture supernatants. The data (Fig. 2) show a direct relationship between the level of \textit{prsA} expression (i.e. IPTG concentration) and the yield of mature rPA.

\textbf{Fig. 2}  

\textit{B. anthracis} possesses three \textit{PrsA} homologues –The unannotated \textit{B. anthracis} genome sequence (The Institute for Genomic Research: http://www.tigr.org) was searched for homologues of \textit{B. subtilis} \textit{prsA}. The genome sequence was translated in all six reading frames and protein-protein alignments were carried out using the blastp algorithm (18). Three \textit{B. anthracis} proteins, named PrsAA, PrsAB and PrsAC, showed homology to \textit{B. subtilis} \textit{PrsA}. Alignment of the \textit{B. subtilis} and \textit{B. anthracis} \textit{PrsA} homologues indicated a high degree of sequence conservation throughout their entire lengths (Fig. 3). The relative identities of \textit{B. anthracis} \textit{PrsA} homologues with \textit{B. subtilis} \textit{PrsA} ranged from 38 – 44\% and the Ppi PPIase I and II domains were well conserved. In contrast, the relative identity between \textit{B. subtilis} \textit{PrsA} and YacD, was 24\%; no YacD homologue was identified in \textit{B. anthracis}.

\textbf{Fig. 3}  

Of the three \textit{B. anthracis} \textit{PrsA} homologues, PrsAA shows the highest similarity (44\%) with \textit{B. subtilis} \textit{PrsA}. Analysis of the region of the chromosome either side of \textit{prsAA} revealed the presence of three genes, \textit{yhal}, \textit{hpr} and \textit{yhaH}, that co-localize with \textit{prsA} of \textit{B. subtilis} (Fig. 4). No genes co-localizing with \textit{B. subtilis} \textit{prsA} were found in the chromosome regions either side of the \textit{B. anthracis prsAB} and \textit{prsAC} genes. This suggests that \textit{B. anthracis prsAA} is the
primary homologue of *B. subtilis prsA*, and that *B. anthracis prsAB* and *prsAC* were acquired after these species separated from a common ancestor.

→ **Fig. 4**

*B. anthracis PrsA homologues are functional in B. subtilis* - The *B. subtilis* PrsA homologue YacD is not able to complement PrsA activity with respect to either viability or secretion (data not shown). We therefore determined whether the *B. anthracis* PrsA homologues could function in *B. subtilis* by establishing a complementation system in which *B. subtilis* and *B. anthracis prsA* genes could be expressed independently. The basis of this complementation system is strain RCW201, in which expression of the native *prsA* is under the control of the IPTG-inducible P_spac promoter. Introduction of xylose-inducible copies of the *B. anthracis* homologues *Ban-prsAA*, *Ban-prsAB* or *Ban-prsAC* into the chromosome of RCW201 at the *amyE* locus (encoding a non-essential α-amylase) generated strains RCW303, RCW304 and RCW305, respectively. The resulting strains expressed either the native *B. subtilis prsA* gene (+IPTG/-xylose), or the *B. anthracis* homologue (-IPTG/+xylose), or both (+IPTG/+xylose). As a control, strain RCW302 was constructed in which a second copy of the *B. subtilis prsA* gene was similarly located at the *amyE* locus under xylose-inducible control.

The ability of individual *B. anthracis prsA* orthologues to complement the essential activity of *B. subtilis prsA* was examined on agar plates, using the strains described above, in the presence of IPTG and/or xylose. The results (Fig. 5) indicated that all three *B. anthracis prsA* homologues are able to complement *B. subtilis prsA* with respect to viability. Moreover, the simultaneous expression of both genes was not detrimental to growth, indicating that the *B. subtilis* and *B. anthracis* PrsA proteins do not interfere negatively with each other. Colonies appearing on media lacking both IPTG and xylose were found to be IPTG-independent and were most probably suppressor mutants (19).
Strain RCW303, carrying Ban-prsAA, exhibited smooth colony morphology in the presence of xylose; conditions favoring PrsA production. This compared with a rough morphology when the host PrsA was produced or co-produced with PrsAA either in the presence of IPTG alone (Fig. 6), or with IPTG and xylose (not shown). In contrast, wild-type B. subtilis, and the strains carrying Ban-prsAB, Ban-prsAC or a second copy of Bsu-prsA, exhibited a normal rough morphology in the presence of xylose (Fig. 6) and/or IPTG (not shown). These observations indicate that Ban-PrsAA has a different substrate specificity to that of the other PrsA proteins.

The influence of B. anthracis homologues on the production of AmyQ and rPA - B. subtilis PrsA levels influence the yield of AmyQ (12) and rPA (Fig. 2). Consequently, strain RCW302 with a xylose-inducible copy of Bsu-prsA, and strains RCW303, RCW304 and RCW305 with xylose-inducible copies of Ban-prsAA, Ban-prsAB and Ban-prsAC, respectively, were used to determine their influence on the secretion of AmyQ and rPA. These strains were transformed with either pKTH10, encoding AmyQ, or pPA101, encoding rPA.

The B. subtilis prsA complementation strains transformed with pKTH10, viz RCW302Amy, RCW303Amy, RCW304Amy and RCW305Amy, were grown in LB broth containing 1% xylose. The growth of the strains expressing Pxyt-controlled B. subtilis or B. anthracis prsA genes was similar to that of RCW101 expressing B. subtilis prsA from its native promoter (Fig. 7). In each case, AmyQ synthesis was induced during transition from exponential to stationary phase and the highest yield of AmyQ was observed in stationary
phase. The stability of AmyQ in culture medium was confirmed by the maintenance of high α-amylase activities in 24 h culture supernatants. RCW101, in which *B. subtilis prsA* was expressed from its native promoter, displayed a yield of AmyQ several-fold higher than the strains in which the *prsA* genes were under xylose regulation. Of the latter strains, RCW302Amy (with *Bsu-prsA*) and RCW304Amy (with *Ban-prsAB*) exhibited a two to three-fold higher yield of AmyQ than strains RCW303 (with *Ban-prsAA*) and RCW305 (with *Ban-prsAC*).

→ Fig. 7

Equivalent experiments were carried out with strains producing rPA, RCW302PA (with *Bsu-prsA*), RCW303PA (with *Ban-prsAA*), RCW304PA (with *Ban-prsAB*), RCW305PA (with *Ban-prsAC*). Again, the growth of strains expressing P_{xyl}-controlled *B. subtilis* and *B. anthracis prsA* genes was similar to that of RCW102 in which the *B. subtilis prsA* was expressed from its native promoter (Fig. 8). In all strains, rPA production peaked during transition to the stationary phase, but then declined with different kinetics, presumably due to the presence of proteases in the culture medium (20). As was seen for AmyQ, the highest rPA yield was observed when *B. subtilis prsA* was expressed from its native promoter, in RCW102, while the maximal rPA yields of strains expressing *B. subtilis* or *B. anthracis prsA* genes from the P_{xyl} promoter were between two- to three-fold lower. Strains expressing *Ban-prsAA* and *Ban-prsAB* produced slightly higher peak yields than those expressing *Bsu-prsA* or *Ban-prsAC*.

→ Fig. 8
DISCUSSION

Lipoprotein PrsA, essential for the viability of *B. subtilis*, is an extracellular chaperone involved in the post-translocational folding of specific secretory proteins. To date, the only secretory protein that has been demonstrated experimentally to require PrsA for folding is AmyQ and mutants with reduced amounts of PrsA show lower α-amylase activities (12). However, as PrsA is required for *B. subtilis* viability, it is likely that at least one protein essential for cell wall synthesis is PrsA-dependent.

We investigated the role of PrsA in the secretion of *B. anthracis* PA, a key component of toxins EdTx and LeTx. Initial studies, using the prsA3 mutation that produces ~10% of the wild-type activity, indicated a role for PrsA in rPA production: unlike the wild-type control, no rPA was detected in the culture supernatant of the prsA3 mutant (Fig.1). The importance of PrsA on rPA production was confirmed using a strain in which the level of prsA expression induced from the P_{spac} promoter was controlled by the addition of IPTG. A 5-fold increase in the concentration of IPTG in the medium (0.5 to 2.5 mM) resulted in a 2.5-fold increase in rPA production (Fig. 2); even at 10 mM IPTG, the amount of rPA was only about half that observed when Bsu-prsA was expressed from its native promoter (data not shown). Together these data indicate that the manipulation of PrsA synthesis could be a useful strategy for increasing the production of rPA.

Since rPA production in *B. subtilis* was PrsA dependent, we were interested to establish whether its native host, *B. anthracis*, encoded a PrsA homologue. Analysis of the raw DNA sequence data unexpectedly revealed the presence of three PrsA homologues in this bacterium, PrsAA, PrsAB and PrsAC. Using a complementation system in which the synthesis of the native *B. subtilis* PrsA or a *B. anthracis* homologue could be controlled independently, we were able to show that all three orthologues were functional in *B. subtilis* with respect to cell viability and protein secretion.
These observations raise the question as to why \textit{B. anthracis} produces three PrsA proteins, while \textit{B. subtilis} produces only one. An explanation might be found in the smooth colonies observed in \textit{B. subtilis} expressing Ban-prsAA, rather than the rough morphology typical of wild-type \textit{B. subtilis} expressing Bsu-prsA or one of the constructs expressing Ban-prsAB or Ban-prsAC. The simplest explanation is that Ban-PrsAA is inefficient at folding a specific PrsA-dependent protein required for normal cellular morphogenesis. It also suggests that individual PrsA proteins are specific for, or can distinguish between, different secretory protein substrates. We attempted to test this hypothesis by analysing the efficiency with which each PrsA protein was able to function in the secretion of two PrsA-dependent proteins, namely AmyQ and rPA (Figs. 7, 8). When individual \textit{B. anthracis} and \textit{B. subtilis} prsA genes were put under the control of the xylose-inducible P\textsubscript{xyI} promoter, AmyQ yields were similarly high in strains expressing Bsu-prsA and Ban-prsAB, while yields from strains expressing Ban-prsAA and Ban-prsAC were significantly lower (Fig. 7). Interestingly, the PrsA homologue that appeared to be the most effective in mediating high AmyQ yields, PrsAB, is that with least amino acid similarity to \textit{B. subtilis} PrsA.

We were not able to ascertain whether the amounts of PrsA synthesised by each of these strains was the same, since the only antibodies that were available were to Bsu-PrsA. While it is reasonable to assume that homologous genes expressed from the same promoter produce similar amounts of their protein, the variations in AmyQ production may reflect differential amounts of functional PrsA due to factors such as: (a) variations in mRNA half-lives of the various prsA genes; (b) variations in the recognition efficiency for the various prsA ribosome binding sites and; (c) differential stability of the various PrsA proteins in \textit{B. subtilis}. Alternatively, the various PrsA lipoproteins may exhibit differential substrate specificities with respect to AmyQ.
In a similar set of experiments rPA yields were examined under conditions when the various \textit{prsA} genes were induced with xylose (Fig.8). rPA levels were generally higher with \textit{Ban-prsAA} and \textit{Ban-prsAC}, and lower with \textit{Bsu-prsA} and \textit{Ban-prsAB}, although the differences in yields were not as marked as with AmyQ. Interestingly, the kinetics of rPA degradation in strains expressing the various \textit{prsA} genes followed different patterns, most likely due to their differential effects not only on the folding of rPA, but on that of the host extracellular proteases which are co-produced in stationary phase. The data (Fig. 8) suggest that the PrsA protein encoded by \textit{Ban-prsAA} increases the persistence of secreted rPA in the medium to a greater extent that either \textit{Bsu-prsA} or the other \textit{B. anthracis} proteins. The decrease in rPA from the maximal concentrations at transition phase is in contrast with the stability and persistence of AmyQ (Fig. 7).

The data for AmyQ, rPA and cell morphology provide clear evidence that the \textit{B. anthracis} PrsA homologues show different but overlapping substrate-specificities. A similar observation has been reported for other components of bacterial protein secretion pathways. A recent analysis of completed bacterial genome sequences (21) has identified nine bacterial species that possess two homologues of SecA: \textit{B. anthracis}, \textit{Listeria monocytogenes}, \textit{Listeria innocua}, \textit{Mycobacteria leprae}, \textit{Mycobacteria smegmatis}, \textit{Mycobacteria tuberculosis}, \textit{Staphylococcus aureus}, \textit{Streptococcus gordonii} and \textit{Streptococcus pneumoniae}. Interestingly, all nine are Gram-positive bacteria that colonise or cause disease in human hosts. That study suggested that \textit{L. monocytogenes} SecA2 might be involved in phase variation, and \textit{secA2} mutants showed a rough, rather than the usual smooth colony morphology and a greatly increased LD$_{50}$ compared with wild-type. At least four of these bacterial species, \textit{B. anthracis}$^{1}$, \textit{Staph. aureus}, \textit{Strept. gordonii} and \textit{Strept. pneumoniae} (22) also encode a second homologue of SecY, namely SecY2, indicating that they have a specialised transporter,

$^{1}$ R.C. Williams, A. Wipat and C.R. Harwood, unpublished results.
SecY2-SecA2, for the export of a subset of secretory proteins. In the case of *Strept. gordonii*, this secondary transporter is required for the transport of a large serine-rich surface protein (GspB) that contributes to platelet binding. Similarly, *B. subtilis* encodes five signal peptidases, SipS, SipT, SipU, SipV and SipW (23). Although all are able to process secretory preproteins, only SipS and SipT are essential for viability: viability is maintained in the presence of either SipS or SipT, but not when both are deleted (24,25). The remaining signal peptidases have a minor role in protein secretion. The endoplasmic reticulum (ER)-type SipW, for example, appears to be required for the processing of two spore-associated preproteins, namely pre-TasA and pre-YqxM (26,27). Again these data indicate that, where they occur, paralogous components of secretory pathways are required for the processing of a subset of protein substrates.

In relation to the *B. anthracis* PrsA homologues, two questions arise. Firstly, when and, in the case of spore-associated proteins, where are these proteins synthesised? Secondly, are individual PrsA proteins associated with specific translocators such as SecY-SecA, SecY2-SecA2 or, possibly, even the twin-arginine transporter (28,29), the genes for which have also been putatively identified in *B. anthracis*².

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² R.C. Williams, A. Wipat and C.R. Harwood, unpublished results.
REFERENCES

1. Pezard, C., Berche, P., and Mock, M. (1991) *Infection & Immunity* **59**, 3472-3477

2. Makino, S., Uchida, I., Terakado, N., Sasakawa, C., and Yoshikawa, M. (1989) *Journal of Bacteriology* **171**, 722-730

3. Mock, M., and Fouet, A. (2001) *Annual Review of Microbiology* **55**, 647-671

4. Avakyan, A. A., Katz, L. N., Levina, K. N., and Pavlova, I. B. (1965) *Journal of Bacteriology* **90**, 1082-1095

5. Belton, F. C., and Strange, R. E. (1953)

6. Leplla, S. H. (ed) (1991) *Anthrax toxin complex*. Source Book of Bacterial Protein Toxins. Edited by Alouf, J. E., and Freer, J. H., Academic Press, London

7. Barnard, J. P., and Friedlander, A. M. (1999) *Infection & Immunity* **67**, 562-567

8. Turnbull, P. C. B. (2000) *Current Opinion in Infectious Diseases* **13**, 113-120

9. Ivins, B. E., and Welkos, S. L. (1986) *Infection & Immunity* **54**, 537-542

10. Wu, X.-C., Wilson, L., Tran, L., and Wong, S.-L. (1991) *Journal of Bacteriology* **173**, 4952-4958

11. Baillie, L. W., Johnson, M., and Manchee, R. J. (1994) *Letters in Applied Microbiology* **19**, 225-227

12. Kontinen, V. P., and Sarvas, M. (1993) *Molecular Microbiology* **8**, 727-737

13. Vitikainen, M., Pummi, T., Airaksinen, U., Wahlstrom, E., Wu, H. Y., Sarvas, M., and Kontinen, V. P. (2001) *Journal of Bacteriology* **183**, 1881-1890

14. Bron, S. (1990) in *Molecular biological methods for Bacillus* (Harwood, C. R., and Cutting, S. M., eds), pp. 75-174, John Wiley and Sons, Chichester

15. Vagner, V., Dervyn, E., and Ehrlich, S. D. (1998) *Microbiology* **144**, 3097-3104
16. Hyrylainen, H. L., Vitikainen, M., Thwaite, J., Wu, H. Y., Sarvas, M., Harwood, C. R., Kontinen, V. P., and Stephenson, K. (2000) *Journal of Biological Chemistry* **275**, 26696-26703

17. Singh, Y., Chaudhary, V. K., and Leppla, S. H. (1989) *Journal of Biological Chemistry* **264**, 19103-19107

18. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) *Journal of Molecular Biology* **215**, 403-410

19. Pragai, Z., and Harwood, C. R. (2000) *Journal of Bacteriology* **182**, 6819-6823

20. Miller, J., McBride, B. W., Manchee, R. J., Moore, P., and Baillie, L. W. (1998) *Letters in Applied Microbiology* **26**, 56-60

21. Lenz, L. L., and Portnoy, D. A. (2002) *Molecular Microbiology* **45**, 1043-1056

22. Bensing, B. A., and Sullam, P. M. (2002) *Molecular Microbiology* **44**, 1081-1094

23. Tjalsma, H., Bolhuis, A., Jongbloed, J. D. H., Bron, S., and van Dijl, J. M. (2000) *Microbiology and Molecular Biology Reviews* **64**, 515-547

24. Bron, S., Bolhuis, A., Tjalsma, H., Holsappel, S., Venema, G., and van Dijl, J. M. (1998) *Journal of Biotechnology* **64**, 3-13

25. Tjalsma, H., Noback, M., Bron, S., Venema, G., Yamane, K., and van Dijl, J. M. (1997) *Journal of Biological Chemistry* **272**, 25983-25992

26. Stover, A. G., and Driks, A. (1999) *Journal of Bacteriology* **181**, 1664-1674

27. Stover, A. G., and Driks, A. (1999) *Journal of Bacteriology* **181**, 5476-5481

28. Jongbloed, J. D. H., Martin, U., Antelmann, H., Hecker, M., Tjalsma, H., Venema, G., Bron, S., van Dijl, J. M., and Muller, J. (2000) *Journal of Biological Chemistry* **275**, 41350-41357
29. van Dijl, J. M., Braun, P. G., Robinson, C., Quax, W. J., Antelmann, H., Hecker, M., Muller, J. P., Tjalsma, H., Bron, S., and Jongbloed, J. D. H. (2002) *Journal of Biotechnology* **98**, 243-254

30. Anagnastopoulos, C., and Spizizen, J. (1961) *Journal of Bacteriology* **81**, 741-746

31. Palva, I. (1982) *Gene* **19**, 81-87

32. Lacey, R. W., and Chopra, I. (1976) *Journal of Medical Microbiology* **7**, 285-297

**FIGURE LEGENDS**

**FIG. 1.** SDS-PAGE analysis of rPA released into culture supernatants during exponential (5.5 h, OD<sub>450</sub> ~ 0.5), early stationary (7 h, OD<sub>450</sub> ~ 3.0) and late stationary phase (21.5 h, OD<sub>450</sub> ~ 4.0) by *B. subtilis* strains encoding wild-type or mutant versions of *prsA*. Lanes: 1, Protein size markers (kDa); 2, MFJ943 (5.5 h); 3, MFJ943 (7 h); 4, MFJ943 (21.5 h); 5, MFJ945 (5.5 h); 6, MFJ945 (7 h); 7, MFJ945 (21.5 h); 8, PA (1 µg); 9, MFJ683 (non-rPA encoding control strain, 21.5 h).

**FIG. 2.** Production of rPA by RCW201PA in the presence of various concentrations of IPTG (0.5 – 10 mM). Quantitation of Western blot data; error bars represent 1 standard error from the mean of triplicate experiments.

**FIG. 3.** Multiple alignments of the protein sequences of *B. subtilis* PrsA and YacD, and *B. anthracis* PrsAA, PrsAB and PrsAC. The protein sequences were aligned using the ClustalW tool (http://www.ebi.ac.uk/clustalw). The PpiC PPIase domains I and II are indicated by the solid and broken boxes, respectively.

**FIG. 4.** The regions of the *B. subtilis* and *B. anthracis* chromosomes encoding *prsA* and homologues. Comparative analysis with the *B. subtilis* genome (http://genolist.pasteur.fr/SubtiList) shows: genes with no *B. subtilis* homologues (*speckled*); genes with *B. subtilis* homologues and showing conservation of gene order with respect to
Bsu-prsA (dark); genes with B. subtilis homologues and showing conservation of gene order at a distal location (light); genes with B. subtilis homologues and showing no conservation of gene order with B. subtilis (no shading). The values on the PrsA homologues represent relative identity (%) with Bsu-PrsA. The values are the start and end of each region are the coordinates (bp) with respect to the nominal start of the respective genomes, as provided at SubtiList (http://genolist.pasteur.fr/SubtiList) and The Comprehensive Microbial Resource (http://www.tigr.org).

FIG. 5. Activity of the B. anthracis prsA orthologues in B. subtilis with respect to their ability to complement the lethal phenotype of prsA. All strains were derivatives of RCW201 (Pspac Bsu-prsA): RCW301 (Pspac Bsu-prsA; Pxyl); RCW302 (Pspac Bsu-prsA; Pxyl Bsu-prsA); RCW303 (Pspac Bsu-prsA; Pxyl Ban-prsAA); RCW304 (Pspac Bsu-prsA; Pxyl Ban-prsAB); RCW305 (Pspac Bsu-prsA; Pxyl Ban-prsAC). Strains were grown in LB broth with 0.1 mM IPTG, washed and then enumerated on LB agar supplemented as follows: unsupplemented (no shading); 1 mM IPTG (diagonal shading); 1mM IPTG and 1% xylose (wavy shading); 1% xylose (horizontal shading); Colonies observed on unsupplemented LB agar were shown to be IPTG-independent (data not shown).

FIG. 6. The influence on B. subtilis colony morphology expressing either B. subtilis or B. anthracis PrsA. Wild-type B. subtilis 168 and complementation mutants RCW302 (Pspac Bsu-prsA; Pxyl Bsu-prsA), RCW303 (Pspac Bsu-prsA; Pxyl Ban-prsAA), RCW304 (Pspac Bsu-prsA; Pxyl Ban-prsAB), RCW305 (Pspac Bsu-prsA; Pxyl Ban-prsAC) were grown on LB agar supplemented with either IPTG (1 mM) or xylose (1%).

FIG. 7. Influence of B. subtilis and B. anthracis PrsA proteins on the yield of α-amylase in culture supernatants. Growth (solid line) and AmyQ yields (broken line) of B. subtilis strains expressing B. subtilis and B. anthracis prsA genes. Bsu-prsA (crosses) was expressed from its native promoter in RCW101. Bsu-prsA (squares, RCW302Amy), Ban-prsAA
(diamonds, RCW303Amy), Ban-prsAB (circles, RCW304Amy) and Ban-prsAC (triangles, RCW305Amy) were expressed from the xylose-inducible promoter, P_xyl. Strains were grown in LB broth with 1 % xylose.

FIG. 8. Influence of *B. subtilis* and *B. anthracis* PrsA proteins on the yield of α-amylase in culture supernatants. Growth (solid line) and rPA yields (broken line) of *B. subtilis* strains expressing *B. subtilis* and *B. anthracis prsA* genes. Bsu-prsA (crosses) was expressed from its native promoter in RCW 102. Bsu-prsA (squares, RCW302Amy), Ban-prsAA (diamonds, RCW303PA), Ban-prsAB (circles, RCW304PA) and Ban-prsAC (triangles, RCW305PA) were expressed from the xylose-inducible promoter, P_xyl. Strains were grown in LB broth supplemented in 1 % xylose.
Table I.

**Bacterial strains and plasmids**

| Strain/plasmid | Relevant genotype/Characteristics | Reference/Source |
|----------------|----------------------------------|------------------|
| B. subtilis    |                                 |                  |
| 168            | *trpC2*                          | (30)             |
| IH6064         | *sacA321*                        | (31)             |
| MFJ683         | IH6064(pUB110)                    | This study       |
| MFJ943         | IH6064::*cat atxA* (pYS5)         | This study       |
| MFJ945         | IH6064::*cat atxA prsA3* (pYS5)   | This study       |
| RCW101         | 168(pKTH10)                      | This study       |
| RCW102         | 168(pPA101)                      | This study       |
| RCW201         | 168 *prsA:: pMUTIN4*              | This study       |
| RCW201PA       | RCW201(pPA101)                    | This study       |
| RCW302         | RCW201 *amyE::pRCW207*            | This study       |
| RCW302Amy      | RCW302 with pKTH10                | This study       |
| RCW302PA       | RCW302 with pPA101                | This study       |
| RCW303         | RCW201 *amyE::pRCW208*            | This study       |
| RCW303Amy      | RCW303 with pKTH10                | This study       |
| RCW303PA       | RCW303 with pPA101                | This study       |
| RCW304         | RCW201 *amyE::pRCW209*            | This study       |
| RCW304Amy      | RCW304 with pKTH10                | This study       |
| RCW304PA       | RCW304 with pPA101                | This study       |
| RCW305         | RCW201 *amyE::pRCW210*            | This study       |
| RCW305Amy      | RCW305 with pKTH10                | This study       |
| RCW305PA       | RCW305 with pPA101                | This study       |

**Plasmids**

| Plasmid        | Relevant genotype/Characteristics                      | Reference/Source          |
|----------------|--------------------------------------------------------|----------------------------|
| pJPR1          | Ap<sup>+</sup>Cm<sup>+</sup> 5’*amyE 3’*amyE, xylose-inducible P<sub>xy</sub> promoter | J. Errington (Oxford University, UK) |
| pKTH10         | Km<sup>+</sup>pUB110 with *amyQ*                       | (31)                       |
| pMUTIN4        | Ap<sup>+</sup>Em<sup>+</sup>Lm<sup>+</sup> integration vector | (15)                       |
| pPA101         | Km<sup>+</sup>pUB110 with *pagA*                       | (9)                        |
| pPA102         | Km<sup>+</sup>pUB110 with *pagA*                       | (9)                        |
| pRCW101        | Amp<sup>+</sup>Em<sup>+</sup>Lm<sup>+</sup>pMUTIN4 with 5’ fragment of *prsA* | This study |
| pRCW207        | Ap<sup>+</sup>Cm<sup>+</sup> 5’*amyE 3’*amyE, xylose-inducible Bsu-prsA | This study |
| pRCW208        | Ap<sup>+</sup>Cm<sup>+</sup> 5’*amyE 3’*amyE, xylose-inducible Ban-prsAA | This study |
| pRCW209        | Ap<sup>+</sup>Cm<sup>+</sup> 5’*amyE 3’*amyE, xylose-inducible Ban-prsAB | This study |
| pRCW210        | Ap<sup>+</sup>Cm<sup>+</sup> 5’*amyE 3’*amyE, xylose-inducible Ban-prsAC | This study |
| pUB110         | Km<sup>+</sup>                                         | (32)                       |
| pYS5           | Km<sup>+</sup>pPA102 with a Gram-negative replication origin | (17)                       |
FIG. 5.
FIG. 6.
FIG. 7.
FIG. 8.
Production of bacillus anthracis protective antigen is dependent on the extracellular chaperone, PrsA
Rachel C. Williams, Mark L. Rees, Myra F. Jacobs, Zoltán Prágai, Joanne E. Thwaite, Leslie W.J. Baillie, Peter T. Emmerson and Colin R. Harwood

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