Characterization of the Sortase Repertoire in *Bacillus anthracis*

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Abstract

LPXTG proteins, present in most if not all Gram-positive bacteria, are known to be anchored by sortases to the bacterial peptidoglycan. More than one sortase gene is often encoded in a bacterial species, and each sortase is supposed to specifically anchor given LPXTG proteins, depending of the sequence of the C-terminal cell wall sorting signal (cwss), bearing an LPXTG motif or another recognition sequence. *B. anthracis* possesses three sortase genes. *B. anthracis* sortase deleted mutant strains are not affected in their virulence. To determine the sortase repertoires, we developed a genetic screen using the property of the gamma phage to lyse bacteria only when its receptor, GamR, an LPXTG protein, is exposed at the surface. We identified 10 proteins that contain a cell wall sorting signal and are covalently anchored to the peptidoglycan. Some chimeric proteins yielded phage lysis in all sortase mutant strains, suggesting that cwss proteins remained surface accessible in absence of their anchoring sortase, probably as a consequence of membrane localization of yet uncleaved precursor proteins. For definite assignment of the sortase repertoires, we consequently relied on a complementary test, using a biochemical approach, namely immunoblot experiments. The sortase anchoring nine of these proteins has thus been determined. The absence of virulence defect of the sortase mutants could be a consequence of the membrane localization of the cwss proteins.

Introduction

Proteins can be covalently attached to the peptidoglycan of Gram-positive bacteria and this binding reaction is catalyzed by membrane-associated transpeptidases called sortases [for review, (1,2,3)]. The anchored proteins contain a characteristic carboxy-terminal sorting signal consisting of a so-called LPXTG motif followed by a hydrophobic domain and a positively charged tail, leading to a transiently membrane associated protein [4]. The enzymatic activity of sortases can be defined as a cleavage between the threonine and the glycine amino acid of the LPXTG motif to generate an acyl-enzyme intermediate which then reacts with an N-terminal amino-acid on the lipid II, regenerating an amide bond. Alternatively, the acceptor may be a protein [1,5,6,7]. Sortase genes are ubiquitous among Gram-positive bacteria. The number of different sortase genes present in a given genome depends on the species [8]. The vast majority of the studied species have between two and seven sortase genes [9]. Sortases from Gram-positive species can be classified into five or four homology groups [2,3,9]. Sortase A is the major sortase, present in the genomes of all Gram-positive bacteria. The other sortases seem to play more defined roles: B sortases anchor proteins involved in iron acquisition, C sortases are involved in pilus polymerization as do B sortases sometimes, and may be required for adaptation to specific niches [10,11,12,13] and D sortases are harbored by species displaying some specific developmental cycle. Therefore, the sortases B, C and D may be expressed at specific times in the bacterial life cycle [2].

This profusion of sortases in bacterial genomes raises questions about sortase repertoires. Previous studies have indicated that sortases recognize their protein substrates specifically and cannot anchor other sortase substrates; it is thus possible to establish theoretical sortase repertoires [9]. However, some motifs appear ambiguous. For example, proteins containing the LPETG or LPKTG motifs may be attributed to the sortase A or sortase C repertoire. In addition, some motifs do not resemble any of the theoretical sortase repertoires [9]. However, some motifs appear ambiguous. For example, proteins containing the LPETG or LPKTG motifs may be attributed to the sortase A or sortase C repertoire. In addition, some motifs do not resemble any of the identified consensus sequences. For example, a QVPTG motif-harboring protein in *S. pyogenes* is a sortase C2 substrate, a class B sortase involved in pilus assembly [14]. Furthermore, when this QVPTG motif is replaced by the LPSTG of a *S. pyogenes* sortase A-anchored protein, sortase A is able to cleave the LPSTG motif, but neither sortase A nor sortase C2 can polymerize the chimera [14]. In that case, the cell wall sorting signal (cwss) is sufficient to determine the specificity of cleavage, but transpeptidation of the...
intermediate requires additional signals. Thus, the motif is not sufficient to determine sortase specificity.

We report here an analysis of B. anthracis cwss proteins and sortase repertoires. B. anthracis is the etiological agent of anthrax (for review, [15]). To note, B. anthracis also possesses an anchoring mechanism by which proteins harboring a SLH domain are non-covalently anchored to the peptidoglycan by means of binding to a pyruvylated polysaccharide [16]. B. anthracis cwss proteins will hereafter refer to proteins that are covalently anchored to the peptidoglycan by the mechanism involving sortases. Based on its genome sequence, this bacterium appears to have three sortases (Srt) — a class A sortase, SrtA, the structure of which has recently been shown to differ from that of other SrtAs [17], a class B sortase, SrtB, and a class D sortase known, and referred to hereupon, as SrtC [18]—and 9 to 11 putative cwss proteins, depending on the analyzed strain [9,19,20]. Interestingly, the triple srtA srtB srtC mutant strains, deleted for all three sortase genes, are not affected in their virulence, independently of the genetic background, toxigenic or encapsulated [21,22].

The in vitro cleavage of synthetic peptides by sortases and the in vivo cleavage and anchoring of some entire cwss proteins in B. anthracis have been reported. A model peptide harboring the KTDNPKGDSEA sequence from the IsdC protein was found to be cleaved only by SrtB in vitro [23]. Similarly, a peptide containing the LPATG motif (as found in BasC) was found to be cleaved by SrtA and a peptide containing the LPNTA motif [as present in BasH and BasI] was found to be cleaved by SrtC only or, to a lesser extent, also by SrtA, depending on the experimental conditions [18,20]. Thus, B. anthracis sortases may display different degrees of specificity for the in vitro cleavage of some peptides. These findings may be a good indication of sortase cleavage specificity, but cannot predict the ability of the sortases to anchor the corresponding protein to the peptidoglycan.

The anchoring of complete proteins in B. anthracis has been studied. SrtA and SrtB anchor BasC and IsdC, respectively [20,23]. SrtC and BasI are encoded by genes in the same operon, containing the LPATG motif (as found in BasC) was found to be cleaved only by SrtB or, to a lesser extent, also by SrtA, depending on the experimental conditions [18,20]. Thus, B. anthracis sortases may display different degrees of specificity for the in vivo cleavage of some peptides. These findings may be a good indication of sortase cleavage specificity, but cannot predict the ability of the sortases to anchor the corresponding protein to the peptidoglycan.

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Bacterial strains, plasmids and growth conditions

Escherichia coli TG1 [28] E. coli HB101 harboring pKK24 were used for cloning and mating experiments [29]. The B. anthracis strains and plasmids used are listed in Table S1. GamR expression under its native regulatory signals was obtained after growing cells in BHI (Difco). pagA-mediated gamR expression was induced by culturing bacteria on BHI plates, for susceptibility tests, and in R medium, for peptidoglycan purification, both supplemented with 0.6% bicarbonate, under an atmosphere containing 5% CO2 (BHI-bic and R-bic) [30,31]. Antibiotics used were spectinomycin (100 μg/ml), kanamycin (40 μg/ml) and erythromycin (for E. coli 150 μg/ml; for B. anthracis 5 μg/ml).

Bacteriophage and susceptibility test

γ phage (laboratory stock) was amplified as described elsewhere [25]. Exponentially growing cultures of B. anthracis cells were spread on plates and 20 μL of a 10^2 dilution of the γ phage solution were spotted onto the surface. Plaques were observed after 16 h of incubation at 37°C.

Construction of recombinant strains

Recombinant strains were constructed as previously described [29,32]. Transduction between B. anthracis strains was mediated by phage CP51 [33].

DNA manipulation

Plasmid extraction, endonuclease digestion, ligation and agarose gel electrophoresis were carried out as described by Maniatis et al. [34]. Polymerase chain reaction (PCR) amplifications were carried out with long-range high-fidelity Taq polymerase (Roche), or with Vent polymerase (Biolabs), according to the manufacturer’s instructions. Plasmid sequences, carried out to check for the absence of mutations on cloned fragments, were determined from PCR products after an amplification step or directly from the plasmid DNA. All sequencing was carried out by Genome Express Sequencing.

Mutant constructs

The gamR-deleted strain was constructed as follows. pGARK20 was obtained by replacing the 1.1-kb Bcl1-Cla1 fragment in pGAR10, with a kanamycin resistant cassette extracted from pAT21 [25,35]. The gamR deletion was excised from pGARK20, by digesting with SplI-EcoRI and inserted into pATAS28, yielding pGARK30. The wild-type gamR allele was replaced with that in pGARK30 after conjugation and a double cross over event into the 7702 strain, giving rise to the 7AG strain. The gamR srtA, gamR srtB, and gamR srtC double mutants were obtained by transduction of the gamR deletion into 7SBON30, 7SBTR30 and 7SBTO30, respectively [25].

Fusion constructs

DNA fragments carrying the cwss-encoding end of the basA, basB, basC, basE, gamR, basF, basG, basJ, basL and basO (Fig. 1) genes
were obtained by PCR amplification from B. anthracis 7702 chromosomal DNA, using the primers basA-5' and basA-3', basB-5' and basB-3', basC-5' and basC-3', basE-5' and basE-3', GamR-5' and GamR-3', basF-5' and bas F-3', basG-5' and bas G-3', basJ-5' and bas J-3', IsdC-5' and IsdC-3', basL-5' and bas L-3', basO-5' and bas O-3', respectively (Table S1).

The GamR-5' primer contains an NdeI site overlapping the ATG initiation codon of the gamR gene. All other 5' primers start with an AgeI site, positioned so as to allow translational fusion with the gamR gene, which contains an AgeI site.

The DNA fragments amplified were inserted into pCR2.1, giving pBasA10, pBasB10, pBasC10, pGamR10, pBasE10, pBasF10, pBasG10, pBasJ10, pIsdC10, pBasL10, pBasO10, respectively.

The digestion of pGamR10 by AgeI/XbaI removed the LPXTG tail sequence from gamR (nt 1646 to nt 1707, codon 1 corresponding to A of the initiation codon). The resulting construct was used for further fusion constructions, in which the LPXTG-charged gamR tail was replaced by the tail of the other 10 proteins.

We obtained pBasB20, pBasC20, pBasL20 by inserting the AgeI/XbaI fragment from pBasA10, pBasC10, pBasH10, pBasL10 into pGamR10 digested with AgeI/XbaI.

pBasB20, pBasE20, pBasJ20, pIsdC20 and pBasO20 by inserting the AgeI/SacI fragment from pBasB10, pBasE10, pBasJ10, pIsdC10 and pBasO10 respectively into pGamR10 digested with AgeI/SacI.

pBasA30, pBasC30 and pBasL30 by inserting the NdeI/XbaI fragment from pBasA20, pBasC20, pBasL20, respectively, into pPPA40 digested with NdeI/XbaI.

We obtained pBasB30, pBasE30, pBasJ30, pIsdC30 and pBasO30 by inserting the NdeI/SacI fragment from pBasB20, pBasE20, pBasJ20, pIsdC20 and pBasO20, respectively, into pPPA40 digested with NdeI/SacI.

We obtained pGamR30 by inserting the NdeI/SacI fragment from pGamR10 into pPPA40 digested with NdeI/SacI.

Complementation constructs

The B. anthracis strains harboring a plasmid encoding the chimeric protein and deletions of the gamR gene and the srtA gene were already resistant to the three antibiotics currently available in our laboratory. The wild-type srtA gene was therefore inserted directly into the plasmid carrying the gamR fusion gene. We ensured SrtA synthesis by creating a new operon, by inserting the srtA gene downstream from the gamR fusion gene stop codon. The DNA fragment carrying the sortase A gene was obtained by PCR amplification from B. anthracis 7702 chromosomal DNA, using the primers CA1-5'/CA2-3'. The 5' ends contain a SacI restriction site followed by a sequence harboring a RBS — GGAG-GAAATATATAA — immediately upstream from the ATG initiation codon of the sortase gene. The fragment was inserted into pCR2.1, giving pCA10. The sortase gene was excised by digestion with EcoRI-SacI and blunted. The srtA gene was inserted into pBasB30, pBasE30 and pBasJ30 digested with EcoRI and blunted, giving pBasB40, pBasE40, pBasJ10, respectively.

The fusion and complementation constructs were transferred by mating experiment into the 7G, 7AG, 7BG and 7CG strains.

GamR production construct

To produce GamR, gamR gene was inserted into pQE30 as follows. gamR was amplified using Bas3' and Bas3' and inserted into pGEM-T-easy, yielding p3367. p3367 was digested by BamHI-HindIII and ligated into pQE30 similarly digested, giving rise to pQE367.

Cell fraction preparation

Cell fractionation was carried out as previously described [37]. Briefly, B. anthracis liquid cultures were centrifuged; supernatant fraction was precipitated with 10% TCA, pellets were sonicated and the soluble fraction (corresponding to the cytoplasm) and insoluble fraction (corresponding to membrane and peptidoglycan fractions) were separated.

Peptidoglycan preparation

Peptidoglycan was prepared essentially as described by [37]. Briefly, cells were grown in 30 mL of R-bic to an OD600 of 1. Ten mL of the supernatant were TCA precipitated. After each subsequent treatment, an appropriate centrifugation was carried out. The pellet was suspended in 5 mL of 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% SDS and boiled for 10 min and 1 mL was put aside (step 1) and the rest centrifuged. The resulting pellet was resuspended in 4 mL and treated similarly; again 1 mL was kept (step 2). The final pellet was resuspended in 3 mL of the same buffer and sonicated twice 30 s (step 3).

Figure 1. Schematic representation of native GamR and 8 chimeric proteins. The sequence encoding GamR from amino-acid one to 550 was fused to each of the 7 "cwss tails"; these are composed of 10 to 23 residues preceding the "cwss" motif, the motif and the native C-terminal end of the studied proteins. Similar constructs with the wild-type GamR and the chimeric protein harboring the pseudo-cwss tail of the non-cwss protein BasO are also represented and highlighted by a rectangle. Numbers indicate number of residues.

doi:10.1371/journal.pone.0027411.g001
The one mL fractions, kept at each step, were centrifuged. The pellets rinsed with 1 mL of the Tris NaCl buffer, resuspended in 100 μL containing 10 units of mutanolysin and digested for 2.5 h at 37°C. Volumes corresponding to the same percentage of the initial pellet were loaded on SDS-gels.

Antibody production

Escherichia coli M15/pREP4/pQE3367 was cultivated and Histagged GamR was purified as described by Qiagen on Ni-NTA resin as described by Qiagen. GamR serum was obtained by subcutaneously injecting 10 μg of Histagged GamR in Freund incomplete adjuvant into OF1 mice (Charles River) four times at 2-week intervals.

Immunodetection

For the Western blot analyses, samples were separated on SDS-12% polyacrylamide gels and the proteins transferred onto nitrocellulose membranes. GamR or chimeric proteins were detected using anti-GamR antibodies diluted at 1/650.

Results

In silico analysis

Our in silico study suggested the existence of 10 cwss proteins in the Ames genome [30] (Table 1). We also found 4 other proteins with LPXTG motifs but unconvincing hydrophobic tails, including that termed BasO. Based on this in silico study and prediction methods [9], we suggested the most likely sortase involved in anchoring these 10 proteins; we assigned the same sortases as Gaspar [20] for 8 proteins and SrtA for BasJ and SrtB for BasL/IsdK/IsdX2 protein (NSKTA motif) (Table 1).

Cwss protein identification

In a previous study, we identified the B. anthracis γ phage receptor, GamR, an LPXTG protein anchored by SrtA [25]. We investigated whether the proteins selected during our in silico study were true cwss proteins, by constructing GamR fusions, in which the cwss tail of GamR was deleted and replaced with “cwss tails” of 8 other proteins, 7 putative cwss proteins and the non-cwss protein BasO, starting at least 10 amino-acid residues before the cwss motif and extending to the native C-terminal end of the studied proteins. (Materials and Methods, Fig.1). The fusion proteins were cloned downstream from the pagA promoter and translation initiation site. Their expression is induced in presence of bicarbonate and CO2 [31].

SrtC and its characterized substrates are normally expressed during stationary phase, but phage lysis can only be assayed during exponential phase [18,24]. Consequently, SrtC substrates, BasH and BasI, were not included in this study.

The eight constructs were transferred to a strain in which gamR was deleted. As a control, gamR was transferred simultaneously into a ΔgamR strain (Table 2). As expected, γ phage lysis was restored upon complementation with GamR [25].

Seven of the 8 strains, in addition to that harboring GamR, displayed susceptibility to the γ phage (Table 2), indicating that these hybrid proteins were accessible to the γ phage. Interestingly, the GamR-BasO hybrid protein did not yield γ phage lysis. We checked that the GamR-BasO fusion protein was indeed produced and determined in which fraction it was present, by western-blotting with anti-GamR antibodies (Fig. 2). When GamR was produced under the control of its native regulatory region, it was, or not, detected, depending on the growth conditions, in the supernatant of the parental strain (Fig. 2A, lane 1, Fig 2B lane 1) and it was present in the cell wall preparations, co-purifying with the peptidoglycan in the parental strain but not the triple mutant strain (Fig. 2B, lane 4 and 8). In contrast to GamR, GamR-BasO was found exclusively in the culture supernatant of the parental strain confirming the absence of anchoring of this chimeric protein (Fig. 2C). This construct served as a negative control; it seemingly validated our assay and indicated that the other seven chimeric proteins were surface exposed.

Table 1. B. anthracis putative cwss proteins and their anchoring sortase.

| Protein name(s) | Ames number | cwss motif | predicted anchoring sortase | In vitro peptide cleavage | In vivo anchorage |
|-----------------|-------------|------------|-----------------------------|--------------------------|-----------------|
| BasA            | BA4346      | LPKTG      | SrtA                        |                          |                 |
| Bas B           | BA0871      | LPATG      | SrtA                        |                          |                 |
| Bas C           | BA5258      | LPATG      | SrtA                        | [20] Ni-NTA chromatography after expression of BasC histidine fusion |                 |
| GamR/BasD       | BA3367      | LPKTG      | SrtA                        |                          | Gamma phage sensitivity [25] |
| BasE            | BA3254      | LPNAG      | SrtA                        |                          |                 |
| BasH            | BA0397      | LPNTA      | SrtC                        | [18] Fluorescent microscopy after expression of fusion proteins [18,47] |                 |
| BasI            | BA5070      | LPNTA      | SrtC                        |                          |                 |
| BasJ            | BA0552      | LGATG      | SrtA                        |                          |                 |
| BasK/IsdC       | BA4789      | NPKTG      | SrtB                        | [23] Cell wall localization after fractionation of an IsdC histidine fusion |                 |
| BasL/IsdK, IsdX2| BA4787      | NSKTA      | SrtB                        |                          | Cell associated localization after crude fractionation [26] |
| BasO            | BA1088      | LPHTG      | NA                          |                          |                 |

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b2-aminobenzoyl-LPXTG motif-diaminopropionic acid was incubated with purified sortase and cleavage is measured by fluorescence.

cIn vivo anchoring of native, GamR, or GFP or histidine fusion proteins, BasC, BasH, BasI, IsdC, by different techniques.

NA: does not apply, no hydrophobic tail.
doi:10.1371/journal.pone.0027411.t001

Bacillus anthracis Sortase Repertoire
Table 2. Repertoire identification.

| cwss tail | Wild-type strain | ΔsrtA | ΔsrtB | ΔsrtC | Predicted sortase | Complementation for lysis | Identified sortase |
|-----------|------------------|-------|-------|-------|------------------|--------------------------|-------------------|
| GamR      | +                | PL+   | IB+   | SrtA   | srtA: yes*       | SrtA                     |                   |
| Bas B     | +                | NT+   | NT+   | NT+   | SrtA             | SrtA                     |                   |
| Bas E     | +                | NT    | NT+   | NT+   | SrtA             | SrtA                     |                   |
| Bas J     | +                | NT    | NT    | NT+   | SrtA             | SrtA                     |                   |
| Bas A     | +                | +     | +     | +     | SrtA             | SrtA                     |                   |
| Bas C     | +                | NT    | NT    | NT+   | SrtA             | SrtA                     |                   |
| Isd C     | +                | NT    | NT    | NT+   | SrtB             | SrtB                     |                   |
| Bas L     | +                | +     | +     | +     | SrtB             | SrtB                     |                   |
| Bas O*    | -                | -     | NT    | NT    | NA               | NA                       |                   |

*PL: Phage Lysis test; + : susceptible; – : resistant to phage lysis.
*IB: copurification with the peptidoglycan as assayed by ImmunoBlot; + : copurifies; – does not copurify.
*+ as shown in Davison et al., 2005.
*Identified by other authors (BasC, [20]); (IsdC, [23]).
*Not a cwss protein.
NT, not tested, NA, does not apply.

Determination of the repertoires of the B. anthracis sortases

To determine which sortase anchored each of the remaining eight (seven chimeric and positive control GamR) proteins, we transferred the fusion constructs into the three gamR mutant strains, each of which has a deletion of one of the three sortase genes. The susceptibility of the different strains is summarized in Table 2.

As expected, in cells producing under its native regulatory signals GamR with its own LPXTG tail, lysis was observed in both srtB and srtC mutant strains but not in the srtA mutant, in agreement with GamR being anchored only by SrtA (Table 2) [25]. This suggested that the phage system may be used to suggest sortase repertoires. We found that the strains containing the three fusion proteins GamR-BasB, GamR-BasE, and GamR-BasJ were resistant to lysis in the srtA mutant and susceptible in the srtB and srtC mutant strains, indicating that they are anchored by Sortase A, the in silico predicted sortase (Table 2). Complementation experiments were carried out to confirm the observed patterns of sortase anchoring for BasB, BasE and BasJ proteins by introducing the wild-type srtA gene into gamR srtA srtB strains expressing the GamR-BasB, GamR-BasE, and GamR-BasJ fusion constructs (see Materials and Methods). Susceptibility to γ phage was restored in all complemented strains confirming that SrtA anchors BasB, BasE and BasJ (Table 2).

In contrast, the four other proteins did not seem to be anchored by, or solely by, the predicted sortase. The srtB strain expressing the GamR-BasA fusion protein was resistant to the phage suggesting that BasA could be anchored by SrtB. All the strains producing GamR-BasC, GamR-IsdC and GamR-BasL were lysed suggesting that the proteins were anchored independently of the present sortases.

Proteins that in an unexpected manner, or always, yielded γ phage lysis

The phage lysis assay on the GamR-BasA harboring strains indicated, in contrast with our and others’ prediction, a sortase B anchoring (Table 1, Table 2). To assess the anchoring further, the covalent binding of the protein to the peptidoglycan was assayed by immuno-detection (Fig. 3A). GamR, when produced in native conditions, was only weakly found in the culture supernatant of the srtB and srtC mutant strains (Fig. 2A, lanes 3, 4). When overproduced, GamR chimeric proteins were found in all supernatants, indicating that the overproduction overwhelmed the anchoring capacity (data not shown and Fig. 3A and 3B lanes 1, 3, 5, 7). Consequently, the presence of chimeric

Figure 2. GamR-BasO is not covalently anchored to the peptidoglycan. Different fractions of GamR (A and B) and GamR-BasO (C) producing strains were analyzed by Western blots. A. Culture supernatant samples of the parental 7702 strain and is sortase mutant derivatives were loaded; lanes 1, 2, 3, srtA; 4, srtB; 5, srtC; 6, purified GamR. B. Peptidoglycan from the parental 7702 (lanes 1 to 4) and the triple mutant srtA srtB srtC (lanes 5 to 8) strains and all the peptidoglycan purification steps were analyzed; lanes, 1, 5, culture supernatants; 2 and 6, 3 and 7, 4 and 8. Peptidoglycan fraction of the purification steps 1, 2 and 3, respectively. These lanes were not all contiguous on the initial gels. C. Cells producing GamR-BasO were grown in R-bic and fractionated; lanes, 1, insoluble (cell surface associated) fraction; 2 soluble (cytoplasm) fraction; 3, culture supernatant.

doi:10.1371/journal.pone.0027411.g002
proteins in the supernatant could not be used as a criterion for absence of anchoring and the peptidoglycan was purified as in the parental strain and step 3 analyzed (see Fig 2B). The presence of GamR-BasA was sought in the peptidoglycan fraction of the parental and each sortase mutant strain (Fig 3A); to note, GamR present in the peptidoglycan fractions migrated more slowly than that in the supernatant. This could be the consequence of peptidoglycan fragments still anchored to the protein. The absence of signal found in the peptidoglycan fraction from the srtA mutant strain and its presence in that of all other strains including the srtB mutant (Fig. 3A lane 4 versus lanes 2, 6 and 8) indicated that, in fact, this chimeric protein is anchored by sortase A. The absence of lysis in the srtA strain could have been due to a protein production problem in the conditions used. We concluded from these results that BasA is anchored by SrtA (Table 2).

Unexpectedly, the phage susceptibility assay suggested that the GamR-BasC, GamR-IsdC and GamR-BasL fusion proteins were phage-accessible in all mutants. Two possibilities could account for that. The first one is that more than one sortase can catalyze the anchoring of these proteins to the peptidoglycan. The second one is that non-anchored but surface accessible proteins can permit phage lysis. BasC and IsdC have been shown to be sortase A- and sortase B- anchored respectively, and were not further studied [20,23]. Consequently, our data suggest that chimeric proteins may be present at the surface in sufficient amount and correct conformation to enable phage lysis even when not covalently anchored to the peptidoglycan. Concerning GamR-BasL, and to discriminate between the two hypotheses, immuno-detection experiments were carried out on proteins extracted from peptidoglycan purified from all GamR-BasL producing strains (Fig. 3B). As with overproduced wild-type GamR, the culture supernatant from all strains contained GamR-BasL chimeric protein. In contrast to the situation observed with GamR or GamR-BasA, a signal was found in the peptidoglycan fraction of all strains. However, a weaker signal was found in the srtB strain than in the others. These data indicate that this chimeric protein was always found surface-associated and suggest that SrtB may be involved in its anchorage.

Discussion

We describe here a system in which the anchoring of the B. anthracis γ phage receptor, GamR, to the peptidoglycan was used as a screen for determining the ability of sortase proteins to anchor B. anthracis cwss proteins to peptidoglycan in vivo. Indeed, previous studies suggested that there are 9 cwss proteins in the B. anthracis Ames strain whereas our in silico analysis suggested that there are 10 cwss proteins (Table 1) [19,20]. Furthermore, in a first attempt to determine the B. anthracis sortase repertoire, Western blots were carried out, as done with Listeria monocytogenes or, more recently, with Streptococcus uberis for instance [39,40], with sera raised against some of the cwss proteins. However, the comparison of the protein present in the different fractions did not enable us to define unambiguously the anchoring sortases. Except for GamR, no obvious signal enhancement was detected in the supernatant of a single sortase mutant strain and thorough purification of the peptidoglycan led to too weak signals (Fig 2A and data not shown).

Unfortunately, the chimeric protein-driven phage lysis approach proved to be too sensitive, phage lysis occurring also in strains in which the proteins were not covalently attached to the peptidoglycan. Coupling this system to immuno-detection of overexpressed proteins, we confirmed some already described anchoring and indicated that sortase A, as predicted, anchored BasA and BasJ. Discrimination based on the motif of the consensus sequence seems efficient.

BasL (IsdK, IsdX2), the status of which was not clear, is covalently anchored to the peptidoglycan (Table 1, [20,26-27,41]). However, because GamR-BasC co-purified with the peptidoglycan independently of the sortases synthesized by the producing strains, we cannot definitively ascribe a sortase to BasL anchoring. Either more than one sortase can catalyze it or, less credible, BasL is not a cwss protein and is anchored by another mechanism. The two motifs NPXTG (IsdC) and NSKTA (BasL) have both been shown to anchor SrtB substrates, namely Lmo2185 (NAKTN) and Lmo2186 (NPKSS) [42]. L. monocytogenes SrtB has the capacity to recognize varied amino acids at position 2 of the sorting motif and prolne is not absolutely required for substrate recognition. Furthermore, in B. anthracis, BasL is in the same operon as IsdC, that is anchored by SrtB, hence simultaneously produced. These proteins belonging to the same physiological pathway, iron metabolism, the involvement of the same sortase is a tempting hypothesis. BasL could yet be, when overproduced, a substrate for SrtA in absence of SrtB: in vivo, the LPNTA motif, that is found in Bas normally anchored by SrtC, is a substrate for SrtA [18,20]. B. anthracis produces 9 or more probably 10 cwss proteins, 6 of which depend on SrtA, 1 or 2 on SrtB, and 2 on the class D sortase, SrtC, for their anchorage.

Phage lysis was obtained with undiluted phage solution in the srtA and in the triple srtA srtB srtC mutant strains when GamR was synthesized under the control of its native promoter and rbs [25]. This suggests that even when produced in native quantities GamR proteins are surface exposed in absence of covalent anchoring to the peptidoglycan. This is in contrast to the situation observed in other bacteria, in which sortase A deleted mutant do not display, at their surface, sortase A substrate, such as InIA in L. monocytogenes, Protein A in Staphylococcus aureus [39,43]. This apparent difference could also be a consequence of the protein quantities synthesized by the bacteria and the detecting methods used. Low amounts of unprocessed form of surface proteins may be undetectable by immuno-reaction. That GamR is surface exposed in B. anthracis srtA mutant strains could be a consequence of B. anthracis lacking efficient LPXTGase activity. LPXTGase are non-ribosomally
synthesized enzymes that cleave LPXTG motifs with a catalytic activity higher than that of sortase [44,45]. B. anthracis could be devoid of LPXTGase or B. anthracis LPXTGase could be less active than that of Streptococcus pyogenes [45]. This could be a selected consequence of B. anthracis SrtA hydrolyzing the LPXTG sorting motif 40 times slower than S. aureus SrtA enzyme [17].

Although std and strB strains displayed attenuated growth in macrophage-like cell line, no virulence defect has been observed with sortase mutant strain in various animal models [18,20,21,22,46]. A possible explanation is that due to the high level of redundancy observed in B. anthracis genome, important functions are not encoded by a single gene, harbored by a single protein [38]. However, given functions may require a precise localization, including surface-association. In that respect, the permanent presence of cew membrane-associated, non-covalently anchored, proteins at the surface of B. anthracis may satisfy this need.

In conclusion, the method relying on phage sensitivity yielded a conclusion similar to the prediction for four sortase A anchored proteins [38]. However, given functions may require a precise localization, including surface-association. In that respect, the permanent presence of cew membrane-associated, non-covalently anchored, proteins at the surface of B. anthracis may satisfy this need.

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Supporting Information

Table S1 B. anthracis strains, plasmids, and oligonucleotides used in this study.

Acknowledgments

We thank S. Dramsi for helpful discussions, M. Moya for critical reading of the manuscript and L. Dur for technical assistance.

Author Contributions

Conceived and designed the experiments: WA SD AF. Performed the experiments: WA SD. Analyzed the data: WA SD AF. Contributed reagents/materials/analysis tools: WA SD AF. Wrote the paper: WA SD AF.
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