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Characterization of the *Bacillus anthracis* S-Layer: Cloning and Sequencing of the Structural Gene

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***Bacillus anthracis*, a gram-positive, spore-forming bacterium, is the etiological agent of anthrax. The gene coding for the S-layer protein (*sap*) was cloned on two contiguous fragments in *Escherichia coli*, and the complete sequence of the structural gene was determined. The protein, Sap, is composed of 814 residues, including a classical prokaryotic 29-amino-acid signal peptide. The mature form has a calculated molecular mass of 83.7 kDa and a molecular mass of 94 kDa on a sodium dodecyl sulfate-polyacrylamide gel. Sap possesses many charged residues, is weakly acidic, and contains only 0.9% methionine and no cysteine residues. The N-terminal region of Sap shares sequence similarities with the *Acetogenium kivui* S-layer protein, the *Bacillus brevis* middle wall protein, the *Thermotoga maritima* Ompα protein, and the *Bacillus thuringiensis* S-layer protein. Electron microscopy observations showed that this S-layer is not observed on *B. anthracis* cells in which *sap* has been deleted.**

Bacillus anthracis, the causative agent of anthrax, is a gram-positive, spore-forming bacterium. Fully virulent bacilli are both capsulated and toxinogenic. The two toxins (lethal and edema toxins) are encoded by the virulence plasmid pXO1, and capsule synthesis is dependent on the presence of a second virulence plasmid, pXO2. When the capsule is absent, the cell wall of *B. anthracis* appears layered and is composed of small fragments displaying a highly patterned ultrastructure (8). Holt and Leadbetter (9) previously described a hexagonal lattice on the surface of vegetative cells of *B. anthracis*. This cell surface structure most likely represents what is called an S-layer, with p6 symmetry and a center-to-center spacing of the particles of 7 to 10 nm.

S-layers, or surface arrays, have been found to be the outermost component of many archaebacteria and eubacteria. In most cases, single proteins compose these structures. When present, they represent 5 to 10% of the total cell protein, implying that their synthesis is energy-consuming for the bacterium. The fact that S-layers are found ubiquitously suggests that they play vital roles in the interaction between the cell and its environment. It has been suggested that the S-layer is an important virulence factor for bacteria such as *Aeromonas salmonicida*, *Campylobacter fetus*, and *Rickettsia* spp., protecting against complement killing, facilitating binding of the bacterium to host molecules, or enhancing its ability to associate with macrophages (see reference 21).

Unlike most S-layers, that of *B. anthracis* is not the outermost component of the virulent bacilli since they are encapsulated; *Azotobacter* spp. are another of the rare examples of bacteria possessing both a capsule and an S-layer. The S-layer may have an important function in linking the capsule to the peptidoglycan wall or controlling the exchange of molecules with the environment. *Bacillus thuringiensis*, a closely related entomopathogen bacillus, possesses an S-layer (12). The *B. thuringiensis* S-layer is composed of linear arrays of small particles arranged with p2 symmetry, the constituent being a 91.4-kDa protein. Another bacillus, *Bacillus brevis*, has an unusual S-layer, since the bacterium is covered by two S-layer proteins, the outer wall protein (molecular mass, 103.7 kDa [25]) and the middle wall protein (MWP) (molecular mass, 114.8 kDa [24]). Transcription of the corresponding operon is initiated from multiple and tandemly arranged promoters (1).

In order to study the *B. anthracis* S-layer, we decided to analyze its composition. We hypothesized that a major cell protein with a molecular mass approximately equal to 94 kDa, which is often observed in high abundancy in culture supernatant fluids of most strains of *B. anthracis*, was the component of the S-layer. Here, we describe the cloning and sequencing of the gene encoding the 94-kDa protein. The relationship between this protein and the surface array component described by Holt and Leadbetter (9) was determined by constructing a *sap* deletion mutant and analyzing it by electron microscopy.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. *Escherichia coli* TG1 (13) was used as a host for plasmid constructions. *E. coli* JM83(pRK24) (23) was used as a host for the mating experiments with *B. anthracis* RP10 (18). The *B. anthracis* plasmidless strain, 9131, was obtained by curing RP31 of pXO1 (19). All these *B. anthracis* strains are derivatives of the Sterne strain, i.e., they are $pXO1^+$ $pXO2^-$. Conjugative transfer from *E. coli* to *B. anthracis* was as described by Pezard et al. (18). Double crossover events were screened for as described by Cataldi et al. (3). *E. coli* was grown in L broth or on L-agar plates (14). *B. anthracis* was grown in brain heart infusion medium (Difco Laboratories) or in SPY medium [60 mM K_2HPO_4, 45 mM KH_2PO_4, 15 mM $(NH_4)_2SO_4$, 10 mM $MgSO_4$, 2.4 mM sodium citrate, 0.2% (wt/vol) glucose, 0.2% (wt/vol) yeast extract]. Spectinomycin was used at 60 μg/ml for *B. anthracis* and *E. coli*, and ampicillin was used at 100 μg/ml for *E. coli.*

DNA manipulations. Methods for the isolation and manipulation of recombinant DNA were as described by Maniatis et al. (13). Chromosomal DNA was prepared as described by Fouet and Sonenshein (6). DNA sequencing was carried out using Sequenase (U.S. Biochemicals) with either the primers provided or an oligomer hybridizing to a determined intragenic sequence.

Oligodeoxynucleotide probes, DNA libraries, and plasmids. The two oligodeoxynucleotide probes used were SEAG1 [5′-GC(TA)GGIAAAAC(TA)TTTC C(TA)GATGTICC(TA)GC(TA)GAT-3′] and SEAG2 [5′-TTC(AT)GTTTT (ATC)CC(ATC)ACTTATT(ATC)ACATC-3′]. In contrast to SEAG1, the SEAG2 oligodeoxynucleotide probe was devised complementary to that directly deduced from the amino acid sequence, the two probes being therefore convergent. DNA libraries were obtained by ligating the product of complete *Hin*dIII and *Eco*RV

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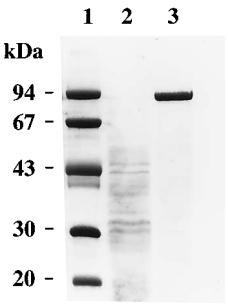

FIG. 1. SDS-PAGE analysis of culture supernatants (1 ml) from different strains of *B. anthracis*. Lane 1, molecular mass markers; lane 2, strain RBA2 (Δ*sap*); lane 3, strain 9131.

digestions of the chromosome to pUC19 previously digested with *Hin*dIII and *Sma*I, respectively, and dephosphorylated. These enzymes were chosen according to the results obtained after hybridizing the two probes to various chromosomal digests. Three of 6,000 *Eco*RV clones and one of 5,000 *Hin*dIII clones were found to hybridize with SEAG1 and SEAG2, respectively. The existence of a common 0.3-kb *Eco*RV-*Hin*dIII fragment between the SEAG1 and SEAG2 hybridizing plasmids, together with the fact that the two probes reacted with two independent *Eco*RV fragments, indicated that the second *Eco*RV fragment should overlap with part of the *Hin*dIII-library plasmid pEAI10 (see Results), a part which was therefore used to screen the *Eco*RV DNA library. Three clones of 250 tested hybridized.

The pEAI207 plasmid was constructed as follows. The internal 1-kb *Hin*dIII fragment from pEAI20 (see Results) was cloned in the conjugative suicide vector pAT113 (23), giving rise to pEAI206. pEAI13 was obtained by subcloning the 0.75-kb *Eco*RV-*Hin*dIII fragment from pEAI10 (see Results) in pUC19 digested by *Sma*I and *Hin*dIII; a spectinomycin resistance cassette, obtained by digesting pUC1318 Spc (15, 22a) with *Sac*I, was inserted in pEAI13, which was similarly digested, creating pEAI204. The *Eco*RI-*Hin*dIII fragment from pEAI204 was rendered blunt and inserted in pEAI206 at the *Sma*I site. This plasmid, pEAI207, therefore harbors a spectinomycin cassette flanked by 1 and 0.75 kb of the coding region of the *sap* gene (i.e., between nucleotides 1005 and 1304 [see Results]).

Hybridization techniques. Colony hybridization and Southern blotting were conducted as described by Maniatis et al. (13). DNA fragments and oligodeoxynucleotides were radiolabeled by nick translation and terminal deoxynucleotidyl transferase, respectively (13).

Protein analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (11). Proteins contained in 1 ml of brain heart infusion medium culture supernatants were precipitated with 10% trichloroacetic acid prior to electrophoresis. In order to establish the N-terminal sequence of the 94-kDa protein, the proteins contained in the supernatant were ammonium sulfate precipitated at 70% saturation. The sequence was then determined from the solubilized pellet with an Applied Biosystems 470A sequencer equipped with a model 120 phenylthiohydantoin analyzer.

Electron microscopy. Bacteria were absorbed onto Formvar-carbon-coated copper grids previously made hydrophilic by glow discharge. They were briefly stained with 1% ammonium molybdate. Excess stain was drawn off with filter paper, and the preparation was allowed to air dry. Observations and recording micrographs were performed on a JEOL 1010 electron microscope operating under standard conditions at either 60 or 80 kV. For the thin sections, pellets of cells harvested by centrifugation were fixed for 30 min at room temperature with 2.5% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.4) containing 0.1 M sucrose, 5 mM $MgCl_2$, and 5 mM $CaCl_2$. Cells were then postfixed for 1 h at room temperature in the above-mentioned buffer, washed with water, and embedded in Spurr's resin. Thin sections were conventionally stained with uranyl acetate and lead citrate and examined with a Philips CM12 electron microscope operating at 80 kV (20).

TABLE 1. N-terminal sequences of proteolytic fragments obtained from the 94-kDa protein

Peptide number(s)	Apparent molecular mass (kDa)	N-terminal sequence[a]
1[b]	94	Ala Gly Lys Thr Phe Pro Asp Val Pro Ala Asp
2, 4	80, 50	Thr Glu Ala Ala Lys Val Glu Ser Ala Lys
3	70	Thr Val Asp Val Asn Lys Val Gly Lys Thr Glu
5	42	Thr Val Val Leu Thr Ala Lys Ser Gly Glu

[a] The underlined residues are identical in the N-terminal sequence of the 94-kDa protein and that of the *B. thuringiensis* S-layer protein.
[b] The mature form of the protein.

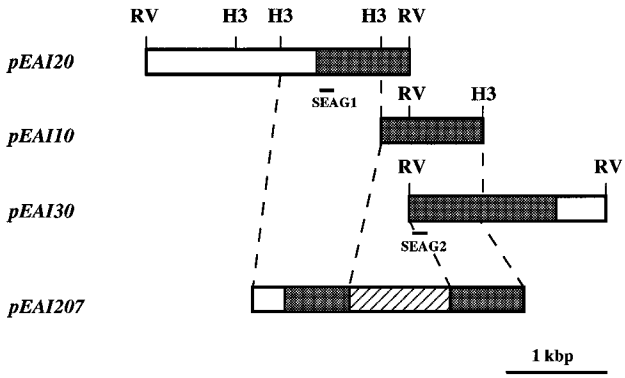

FIG. 2. Restriction map of plasmids containing contiguous fragments of the *sap* gene. The vector for pEAI10, pEAI20, and pEAI30 is pUC19, whereas that for pEAI207 is pAT113. RV, *Eco*RV; H3, *Hin*dIII. The dark boxes represent the coding regions, and the hatched one represents the spectinomycin resistance cassette. The probes used are indicated, and a scale bar is shown.

RESULTS

Cloning the S-layer gene in *E. coli*. Since the S-layer protein is often the most abundant protein produced by a number of different bacterial species, we presupposed that a major cell protein which migrates with an apparent molecular mass of 94 kDa on an SDS-polyacrylamide gel could be a component of the *B. anthracis* S-layer. This 94-kDa protein is produced by various *B. anthracis* strains, including plasmidless strains, and must therefore be chromosomally encoded. In the culture supernatants of *B. anthracis* 9131, the 94-kDa polypeptide may represent 90% of the protein in the medium (Fig. 1). This is not inconsistent with it being a component of the S-layer, since organisms like *B. brevis*, *B. thuringiensis*, and *Caulobacter crescentus* shed excessive S-layer material into the growth medium (2, 12, 22). Since *B. anthracis* and *B. thuringiensis* are closely related, we initially compared the N-terminal sequence of the 94-kDa protein with the known N-terminal sequence of the *B. thuringiensis* S-layer protein (12). The N-terminal sequences of the two mature proteins were identical to one another in 7 of the first 8 residues (underlined in Table 1). The 94-kDa protein present in the culture supernatant of *B. anthracis* was directly subjected to limited chymotrypsin digestion. The N-terminal sequences of the resulting four major polypeptides are shown in Table 1. It is noteworthy that fragments 2 and 4 possessed the same sequence, indicating that the latter may have been derived from fragment 2 by C-terminal proteolysis.

From two of the polypeptide sequences, oligonucleotide probes were synthesized by using the codon bias towards A and T nucleotides found in known *B. anthracis* genes. With these probes (SEAG1 and SEAG2), two overlapping fragments were cloned from chromosomal DNA libraries, yielding the plasmids pEAI20 and pEAI10 (Fig. 2). pEAI30 (Fig. 2) was obtained by screening the *Eco*RV DNA library with a fragment of pEAI10. The cloned gene was named *sap* (for surface array protein).

Sequence analysis. The sequence of *sap* contained one open reading frame (ORF) of 2,442 nucleotides (Fig. 3). The ORF was preceded by a Shine-Dalgarno sequence (AAGGAGG) with a calculated ΔG of -17.8 kcal. There was an 11-nucleotide spacing between the last G of the Shine-Dalgarno se-

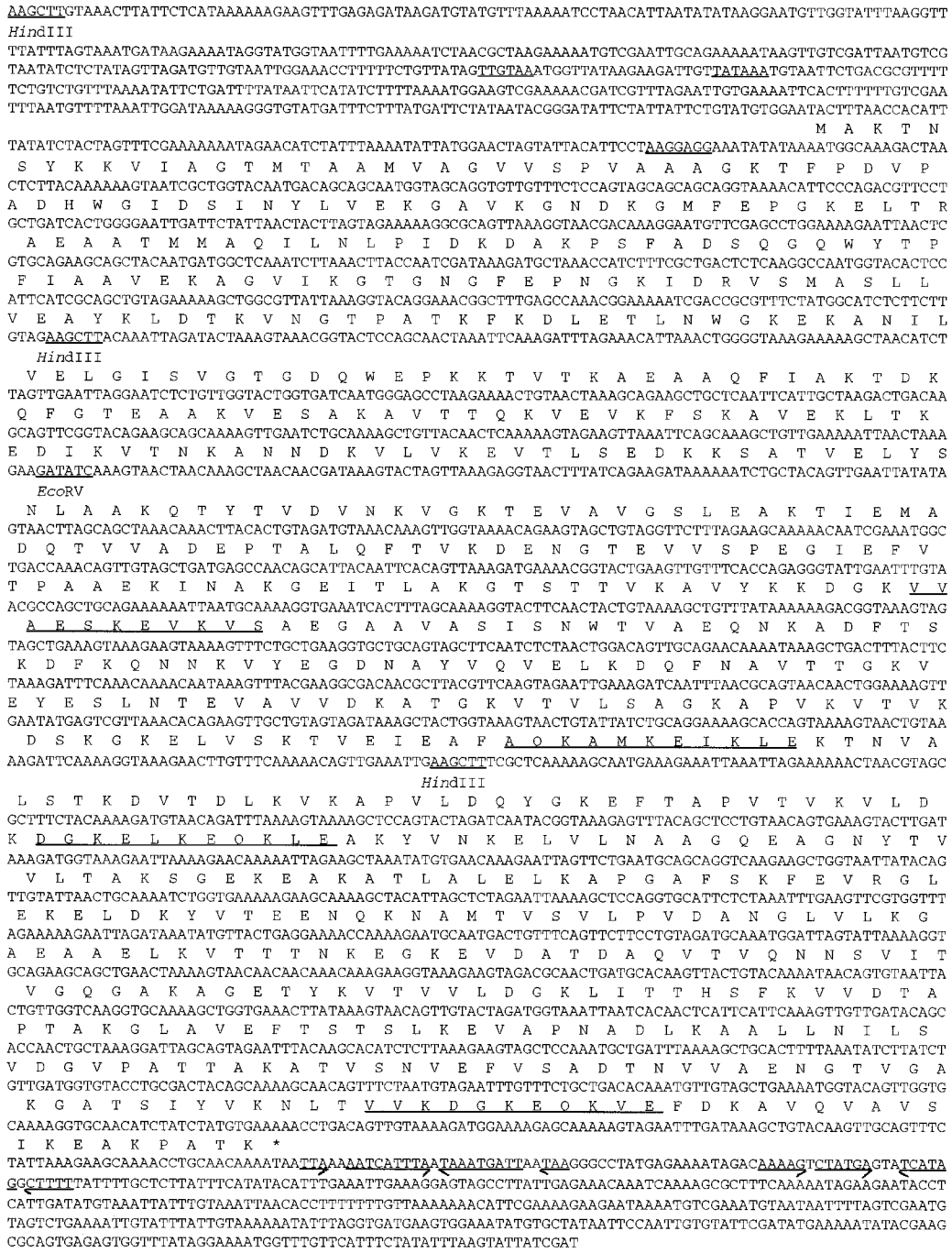

FIG. 3. DNA and deduced amino acid sequence of the region encompassing the *sap* gene. In the left margin, numbers in roman type indicate the first nucleotide of the line, and numbers in italic type refer to the amino acid residues, for which the one-letter code has been used (the asterisk represents the stop codon). The putative ribosome-binding site and promoter sequences as well as the four short internal repeats, have been underlined. The arrows indicate the palindromic structures. The *Hin*dIII and *Eco*RV restriction sites shown in Fig. 2 are indicated.

quence and the ATG initiation codon that is consistent with that observed in other gram-positive bacteria (26).

Two palindromic structures were observed downstream from the TAA stop codon, the first beginning just after the TAA ($\Delta G = -11.6$ kcal) and the second beginning 49 nucleotides farther downstream ($\Delta G = -6$ kcal) followed by a stretch of U's. These two sites may correspond to a factor-independent transcription termination signal.

Translation of the ORF produced an 814-amino-acid protein with a calculated molecular mass of 86,567 Da. The sequences of the chymotryptic polypeptides were localized to various positions throughout the protein (Fig. 3). A comparison of the N terminus of the mature protein (Table 1) and the amino acid sequence deduced from the nucleotide sequence (Fig. 3) indicated that this protein is synthesized as a pre-polypeptide with a 29-amino-acid signal peptide. The signal

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371     VVAESKEVKVS
490     AQKAMKEIKLE
540     DGKELKEQKLE
785     VVKDGKEQKVE
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FIG. 4. Alignment of the four short internal repeats of the Sap protein. Identities (bold characters) and similarities (I, L, V, M; D, E; underlined characters) are indicated whenever two repeats share such a feature.

peptide is similar to other gram-positive signal peptides, with a charged N terminus (3 lysine residues) followed by a hydrophobic domain, a proline residue, and an alanine-rich region encompassing the cleavage site. The presence of the signal sequence implies that the mature form of the protein has a molecular mass of 83,720 Da. On an SDS-polyacrylamide gel, however, Sap migrates with an apparent molecular mass of 94 kDa, suggesting that it may be glycosylated, as has been previously described for other S-layer proteins (21).

The amino acid composition of the Sap protein was compared with that of other S-layer proteins. It is noteworthy that all these proteins share a number of common features. For example, they are devoid of cysteine residues, contain very few methionines (0.9% in the mature form of the *B. anthracis* Sap protein), and have similar percentages of nonpolar, acidic, and basic residues (21). Like the *Acetogenium kivui* S-layer protein (16), the *B. anthracis* Sap protein is not very acidic, with a calculated pI of 6.02. This is in contrast to other S-layer proteins that tend to have a more acidic pI.

S-layer proteins often harbor internal repeats, and two types were found in Sap. A short sequence was repeated four times throughout the second half of the protein (Fig. 3 and 4). It was lysine and glutamic acid rich and had a conserved motif (6 of 11 residues were identical or similar) (Fig. 4). A longer repeat was present twice and was localized in the N-terminal part of the protein, as has been observed for other S-layer proteins (Fig. 5).

Several proteins sharing similarities with Sap were found by using the FASTA program of the Genetics Computer Group package to scan the SwissProt database. The proteins with the best initial scores were the S-layer protein of *A. kivui* (16) with 15.3% identity and the Ompα protein of *Thermotoga maritima* (4) that exhibited 18.5% identity in a 346-amino-acid overlap. The greatest similarity was found between the N-terminal regions of these proteins (Fig. 5). The Pileup program was applied to Sap and five other sequences because of shared similarities, as suggested by the FASTA search, or as indicated by other authors. The proteins compared were Sap, the *A. kivui* S-layer protein (16), the MWP of *B. brevis* (24), the hexagonally packed intermediate layer protein of *Deinococcus radiodurans* (17), the Ompα protein of *T. maritima*, and the outer wall protein of *B. brevis* (25). Pileup indicated that the *A. kivui* S-layer protein and the *B. brevis* MWP are the most similar, immediately followed by the *B. anthracis* Sap and the *T. maritima* Ompα. Interestingly, while overall similarities were not considerable, the N-terminal regions aligned well. The greatest similarities were between regions which correspond to internal repeats found in the S-layer proteins and overlap the longer Sap internal repeat (Fig. 5).

Characterization of the *sap* gene product. To study the role of the S-layer structure, a *sap* deletion mutant was constructed. The *Hin*dIII-*Eco*RV fragment between nucleotides 1005 and 1304 (pEAI 207 [Fig. 2]) was removed and replaced by a spectinomycin resistance cassette on a conjugative suicide vector. Subsequently, the wild-type *sap* gene was deleted by allelic exchange on the chromosome of RP10 which was subsequently cured of pXO1. The secreted proteins produced by the resulting *sap*-deleted strain (RBA2) were compared with those in the supernatant of strain 9131. On an SDS-polyacrylamide gel, there was an absence of the 94-kDa protein in RBA2 (Fig. 1).

We examined the correlation between the presence of Sap and an S-layer around *B. anthracis*. The electron micrographs of strains 9131 and RBA2 are shown in Fig. 6. The S-layer was visible around the 9131 bacterium, with a typical patterned ultrastructure, but was not observed around RBA2. This supports the notion that the *sap* gene encodes a protein associated with the *B. anthracis* S-layer. Colonies of RBA2 and 9131 were morphologically very different. The RBA2 colonies were much larger than the colonies of 9131, and they tended to connect when streaked. When the two strains were grown in SPY liquid medium, the Δ*sap* strain flocculated during growth and sedimented as soon as shaking was arrested, which is different from the behavior of the wild type. Similar morphological changes were observed with other constructed mutants deficient in Sap production. Analysis with an optical microscope showed that cells from 9131 were classical rod-shaped bacilli (≅5 μm long), whereas RBA2 cells were long and filamentous (≅100 μm long). The presence of these long filaments could explain the flocculation observed in liquid culture and the formation of large colonies. It is tempting to correlate all these morphological differences with the variations in Sap protein quantity, i.e., with the presence or absence of an S-layer.

DISCUSSION

In a first step to study *B. anthracis* S-layer structure, the gene encoding the corresponding protein subunit was isolated on

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                                          ↓
Bb    1        MKKVVNSVLASALALTVAPMAFAAEEAATTTAPKMDADMEKTVKRLEALGLVAGYGNGEYGV
Ak    1     MKNLKKLIAVVSTFALVFSAMAVGFA..ATTPFTDVKDDAPYASAVARLYALNITNGVGDPKFGV
Tm    1        MKRVL...LTVAMLSVFFSAMFA.....FFPDVPKDHHWAYEYVWKLWQRGIFIGYPDGEFKG
Ba    1 MAKTNSYKKVIAGTMTAAMVAGVVSPVAA..AGKTFPDVPADHHWGIDSINYLVEKGAVKGNDKGMFEP
Ba  170                                                  YKLDTKVNGTGTGD.QWEP

Bb   63 DKTITRAEFATLVVRARGLEQGAKLAQFSN.TYTDVKSTDWFAGFVNVASGEEIVKGFPDKSFKPQNQ
Ak   64 DQPVTRAQMITFVNRMLGYEDLAEMAKSEKSAFKDVPQNHWAVGQINLAYKLGLAQGVGNGKFDPNSE
Tm   55 DRYITRYEAATAVSRLLDFIEQKMLAG.ASGDLAQV.VGNLSDKYMALEEKVNGLTGILDTL...ASQ
Ba   67 GKELTRAEAATMMAQILNLPIDKD....AKPSFADS.QGQWYTPFIAAVEKAGVIKGTGNG.FEPNGK
Ba  188 KKTVTKAEAAQFIAKTDKQFGTEA....AKVESAKA
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FIG. 5. Comparison of the N-terminal region of the Sap protein with that of two other S-layer proteins and one related protein. The four sequences (*B. brevis* MWP, *Bb*; *A. kivui* S-layer protein, *Ak*; *T. maritima* Ompα protein, *Tm*; and *B. anthracis* Sap protein, *Ba*) were aligned by introducing gaps (dots). The Sap protein internal repeat, starting at residue 170, was added. For the sake of clarity, the two *B. anthracis* sequences have been grouped at the bottom of the figure, whereas the Pileup program had inserted the N-terminal sequence of Sap between the *A. kivui* and the *T. maritima* sequences. The signal peptide cleavage sites (arrow) have been aligned. Identity and similarity (Fig. 4; also, A, G; D, E, N, Q; F, W, Y; S, T, K, R) were established in comparison with the N-terminal portion of the Sap protein.

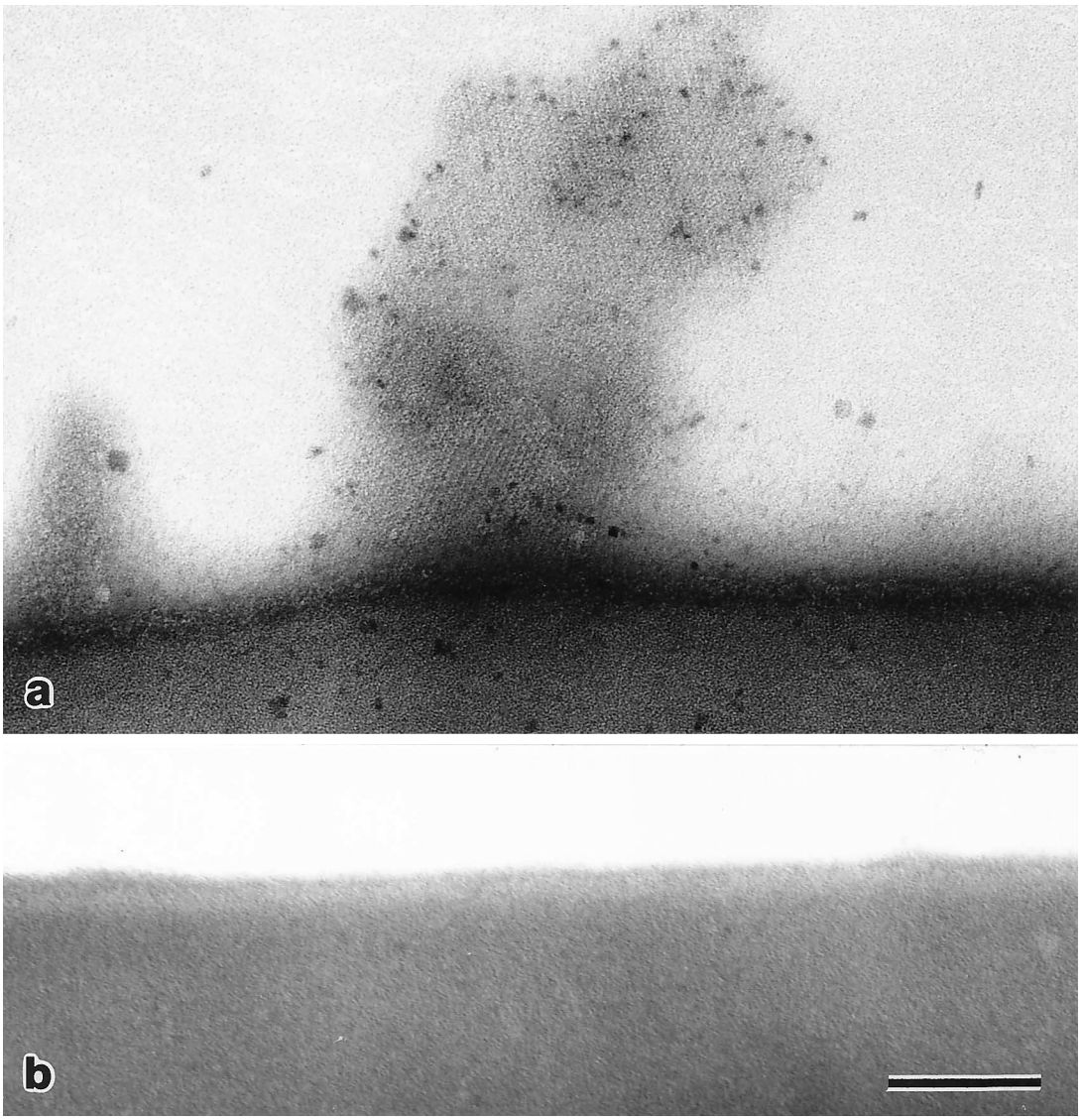

FIG. 6. Electron micrographs of the cell walls of *B. anthracis* cells. Cells were negatively stained with ammonium molybdate. (a) Sap^+ strain 9131; (b) Sap^- mutant strain RBA2; (c) ultrathin section of intact cell of Sap^+ strain 9131; (d) ultra thin section of intact cell of Sap^- mutant strain RBA2. s, S-layer; pg, peptidoglycan; cm, cytoplasmic membrane. Bar, 0.1 μm.

two contiguous DNA fragments. Oligonucleotide probes derived from established amino acid sequences of the 94-kDa major extracellular protein were successfully used to clone the corresponding gene (*sap*). Sequence data and deletion analysis support the notion that the S-layer comprises the 94-kDa protein.

Sequences resembling the recognition sequences of *Bacillus subtilis* σ^A factor were looked for in the region upstream from this ORF, and none which were really convincing were found. This absence is not entirely surprising. Such sequences have not been found for *B. anthracis* promoters where transcription is initiated at a high level, for example, upstream from a toxin component gene (10). Moreover, the promoter, which is probably responsible for the constitutive high-level synthesis of the *B. brevis* cell wall proteins, lacks the −35 sequence (1). The absence of a σ^A consensus sequence in the vicinity of the known *B. anthracis* genes could be due to altogether different σ factor recognition sequences. This would be unusual, however, since phylogenetically more distant organisms, such as *E. coli* and *B. subtilis*, share the same consensus sequences. Another explanation is that these *B. anthracis* genes are not transcribed by a major σ factor, although less utilized σ factor recognition sequences were not found. The hypothesis that we favor is that these genes are positively regulated and that, as with such genes from *B. subtilis*, an activator compensates for the poor recognition sequence. The regulatory region of the *B. anthracis sap* gene awaits further analysis.

The amino acid sequence of Sap was compared with those of other S-layer proteins. Sequence similarities were found for the N-terminal regions of Sap, two other S-layer proteins (MWP from *B. brevis* and the S-layer protein from *A. kivui*), and the *T. maritima* Ompα protein. These sequences contain internal repeats that could play an important role in the assembly of the layer. Interestingly, proteins implicated in the attachment of *Clostridium thermocellum* cellulosome to the membrane also have similarities with the N-terminal repeats

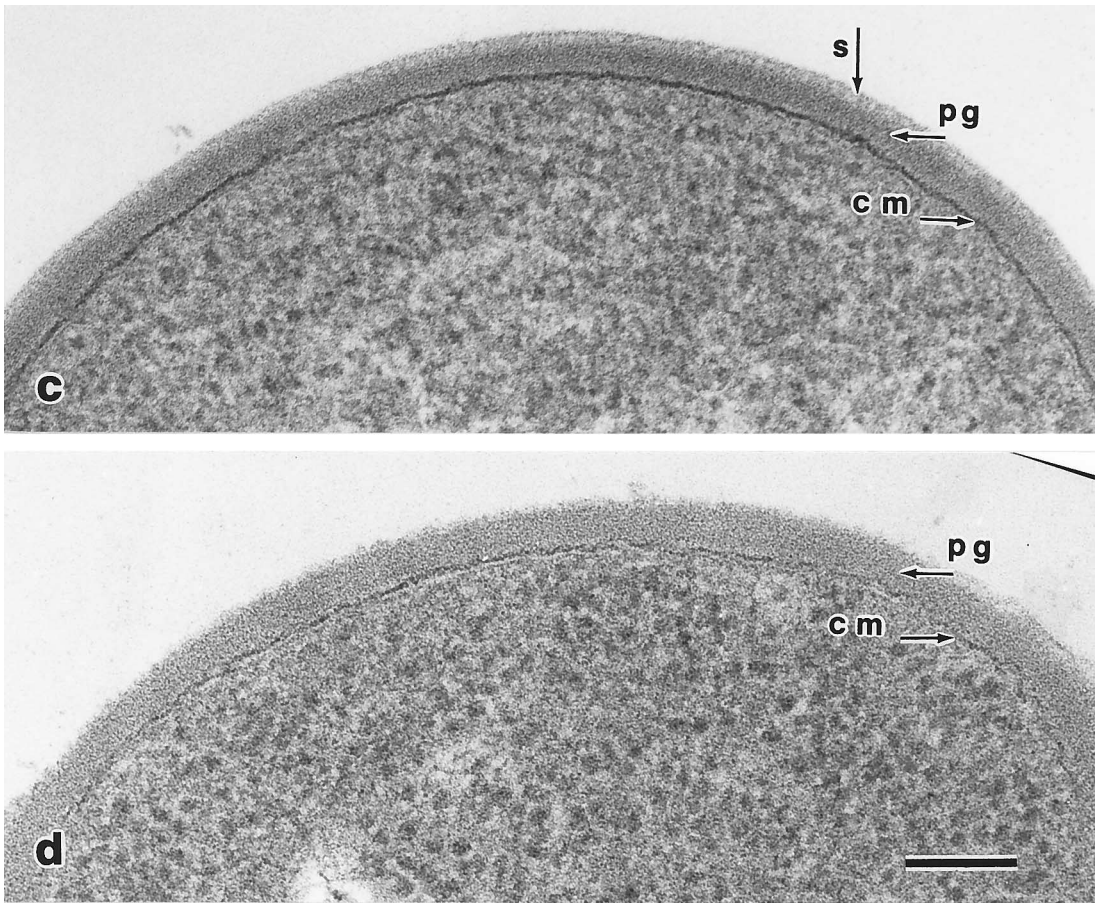

FIG. 6—*Continued*.

from the S-layer proteins (7). Therefore, these repeat regions may be important in the interaction between the peptidoglycan wall and the S-layer structure. Moreover, a highly charged sequence was found to be repeated four times in Sap. It could have an important structural role. It has been suggested that the *T. maritima* Ompα protein connects the outer membrane to the inner cell body, with its carboxy-terminal hydrophobic tail probably being buried in the membrane. Therefore, the *B. anthracis* Sap protein may anchor the capsule to the peptidoglycan wall, utilizing both its repeated sequences and its hydrophobic C-terminal tail.

The function and the in vivo expression of the *B. anthracis* S-layer remain unknown. In the absence of the S-layer, dramatic morphological changes are observed, under both liquid and solid culture conditions. Since synthesizing the S-layer is energy-consuming, it would be interesting to determine where the pressure to maintain it occurs: in the soil or in the host. A major cell-associated protein with a molecular mass of 91 kDa (EA1) has been reported to be a predominant antigen following vaccination with the live spore vaccine (5). It should now be of interest to determine if EA1 corresponds to the Sap protein. If the host is where the S-layer is maintained, its role cannot be simply to protect against complement killing or to enhance the binding of the bacterium to host molecules or macrophages, since in vivo the bacteria are encapsulated. Therefore, although *B. anthracis* probably goes through few, if any, life cycles in the soil, an eventual protective role against osmotic pressure could exist in this environment where the S-layer could be the outermost wall structure. In the future, an extensive study of the synthesis of the S-layer in vivo as well as that of an eventual interaction between the S-layer and the capsule will have to be undertaken.

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