A general strategy for identification of S-layer genes in the *Bacillus cereus* group: molecular characterization of such a gene in *Bacillus thuringiensis* subsp. *galleriae* NRRL 4045

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Despite its possible role in virulence, there has been little molecular characterization of members of the S-layer protein family of the *Bacillus cereus* group. It is hypothesized that the components of the S-layers are likely to display similar anchoring structures in *Bacillus thuringiensis* and *Bacillus anthracis*. Based on inferred sequence similarities, a DNA fragment encoding the cell-wall-anchoring domain of an S-layer component of *Bac. thuringiensis* subsp. *galleriae* NRRL 4045 was isolated. The complete gene was identified and sequenced. An ORF of 2463 nt was identified, which was predicted to encode a protein of 821 aa, SlpA. The mature SlpA protein (792 residues) carries three S-layer homology (SLH) motifs towards its amino terminus, each about 50 aa long. These motifs were sufficient to bind *Bac. thuringiensis* and *Bac. anthracis* cell walls *in vitro* by interacting with peptidoglycan-associated polymers, confirming a common wall-anchoring mechanism. The *slpA* null-allele mutant was constructed and shown to possess no other abundant surface protein. It is proposed that the method described in this paper could be applied to the identification and deletion of any *Bac. cereus* S-layer gene and is of great value for the molecular and functional characterization of these genes.

Keywords: SLH motifs, cell-wall anchoring, surface antigens, Gram-positive

INTRODUCTION

The cell surface of many archaea and eubacteria is covered by a crystalline array known as the surface layer (S-layer). This macromolecular structure results from the non-covalent, entropy-driven self-assembly of proteins (Sára & Sleytr, 2000). Most arrays consist of a single protein species, with one well-documented exception, *Clostridium difficile*, in which the S-layer consists of two different proteins in equimolar proportion (Takeoka *et al.*, 1991; Cerquetti *et al.*, 2000). In some species such as *Bacillus anthracis*, *Brevibacillus brevis*, *Aquaspirillum serpens*, *Bacillus stearothermophilus* and *Campylobacter fetus*, more than one S-layer protein may be present at the surface of the bacterium. The S-layer proteins may be synthesized simultaneously

(e.g. *Bre. brevis*, *A. serpens*) or sequentially (e.g. *C. fetus* and *Bac. stearothermophilus*) (for review, see Sára & Sleytr, 2000). These proteins interact with underlying cell-wall polymers via non-covalent bonds (Boot & Pouwels, 1996; Ries *et al.*, 1997; Mesnage *et al.*, 2000).

During the last decade, several S-layer genes have been cloned and sequenced (for review, see Sára & Sleytr, 2000). The deduced primary structures of the proteins encoded by these genes share common features (a very low content of sulphur-containing amino acids, large amounts of aspartic and glutamic acid or a high proportion of hydrophobic residues), but no extensive sequence similarity has been found between them, except in domains involved in cell-wall targeting, i.e. SLH domains (Lupas *et al.*, 1994). Therefore, no relationship has been identified between sequence and structural organization of the S-layer. Few data are available concerning the function of S-layers. Nevertheless, some studies have shown that the S-layer is involved in cell integrity and shape maintenance in

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archaea, in which it is often the only cell-envelope component (Mescher & Strominger, 1976; Wildhaber & Baumeister, 1987). In some pathogenic bacteria, the S-layer is involved in resistance to complement-mediated killing or interaction with host cells and molecules (Pei & Blaser, 1990; Kotiranta *et al.*, 1998). In addition, S-layers are often the outermost cell-envelope component and are thought to be involved in the regulation of macromolecule exchange with the environment, by acting as a molecular sieve (Sára & Sleytr, 1987; Sára *et al.*, 1992).

S-layer proteins are well documented in the genus *Bacillus*, but not so well in the *Bacillus cereus* group (for review, see Sidhu & Olsen, 1997). The *Bac. cereus* group comprises four species: *Bac. cereus*, *Bacillus thuringiensis*, *Bac. anthracis* and *Bacillus mycoides* (Priest, 1993). *Bac. cereus*, *Bac. thuringiensis* and *Bac. anthracis* have recently been proposed as a single species (Helgason *et al.*, 2000). Some members of the *Bac. cereus* group are pathogens: *Bac. thuringiensis* is an entomopathogen commonly used in agriculture, *Bac. cereus* is a soil bacterium and an opportunistic human pathogen and *Bac. anthracis* is the causative agent of anthrax, a disease of mammals. Previous studies suggest that the *Bac. cereus* S-layer may promote interactions with human polymorphonuclear leucocytes, as well as interactions with host (Kotiranta *et al.*, 1998). Therefore, S-layer proteins may contribute to the pathogenicity of some bacilli. To date, no S-layer mutant has been produced in the *Bac. cereus* group, except in *Bac. anthracis* (Etienne-Toumelin *et al.*, 1995; Mesnage *et al.*, 1997). In that organism, there are two S-layer proteins, Sap and EA1, and their genes were disrupted independently and simultaneously (Etienne-Toumelin *et al.*, 1995; Mesnage *et al.*, 1997). However, the function of the S-layer as a virulence factor has not been investigated. Indeed, in *Bac. anthracis* the S-layer is covered by a polypeptide capsule *in vivo*, rendering the precise definition of its function difficult (Mesnage *et al.*, 1998). To investigate the role of the S-layer in bacteria from the *Bac. cereus* group, other than *Bac. anthracis*, we devised, and tested on a given example, a strategy that could be of general use for constructing null mutants in the S-layer-encoding genes, the last step being an allelic exchange. When this work was initiated, only one *Bac. cereus* group S-layer had been thoroughly studied, that of *Bac. thuringiensis* subsp. *galleriae* NRRL 4045, for which biochemical characterization had been reported (Luckevich & Beveridge, 1989).

In this paper, we report the cloning of an S-layer gene of *Bac. thuringiensis* subsp. *galleriae* NRRL 4045 and the construction of a mutant deficient in S-layer protein synthesis as a means of defining a general method for any *Bac. cereus* strain. We also analysed the cell-wall targeting mechanism of this protein. We found that the SLH domain interacted with peptidoglycan-associated polymers of *Bac. thuringiensis* and *Bac. anthracis*. The S-layer gene cloning and disruption method reported here is general and may be applied to many other *Bac. cereus* genes encoding S-layer proteins.

METHODS

Bacterial strains, vectors and culture media. *Escherichia coli* TG1 (Sambrook *et al.*, 1989) was used as a host for cloning. *E. coli* HB101 harbouring pRK24 (Trieu-Cuot *et al.*, 1987) was used for mating experiments. *Bac. thuringiensis* subsp. *galleriae* NRRL 4045 was provided by T. J. Beveridge (University of Guelph, Canada). In *Bac. anthracis* SM11, both *Bac. anthracis* S-layer components have been deleted (Mesnage *et al.*, 1997).

For cloning experiments, plasmids pUC19, pNEB193 and pQE30 were routinely used (Yanisch-Perron *et al.*, 1985; New England Laboratories; Qiagen). For mating experiments, DNA fragments were inserted into pAT113 (Trieu-Cuot *et al.*, 1991).

E. coli was cultured in L-broth or on L-agar plates (Miller, 1972). *Bac. thuringiensis* and *Bac. anthracis* cells were grown in BHI (Difco) broth or on BHI-agar plates. Antibiotics were used at the following concentrations: 100 µg ampicillin ml⁻¹ and 40 µg kanamycin ml⁻¹ for *E. coli*; 100 µg spectinomycin ml⁻¹ and 300 µg spectinomycin ml⁻¹ for *E. coli* and *Bac. thuringiensis*, respectively.

DNA manipulation and sequencing. Methods for plasmid extraction, endonuclease digestion, phosphorylation with T4 polynucleotide kinase, ligation and agarose electrophoresis were as described by Sambrook *et al.* (1989). PCR amplification and the filling-in of the ends of DNA molecules with Vent DNA polymerase were performed as indicated by the manufacturer of the enzyme. If bacterial colonies were used instead of DNA, the polymerase was added after the initial incubation for 5 min at 100 °C. Chromosomal DNA was extracted as described by Delecluse *et al.* (1991). Sequences were determined from PCR products or from double-stranded DNA, by the dideoxy chain termination procedure (Sanger *et al.*, 1977) using the prism AmpliTaq Dye Primer sequencing kit (Applied Biosystems) with an ABI PRISM 373A sequencer. Nucleotide and deduced amino acid sequences were analysed using the GCG Wisconsin package (version 9.1; Genetics Computer Group) and the CLUSTAL W program (version 1.8).

General methods. *E. coli* cells were made competent as described by Chung & Miller (1988). Recombinant plasmids were transferred from *E. coli* to *Bac. thuringiensis* by heterogramic conjugation (Trieu-Cuot *et al.*, 1987). Allelic exchange was carried out as described previously (Pezard *et al.*, 1991).

Cloning and disruption of the *slpA* gene. The initial DNA fragment (about 620 bp) was isolated by PCR with Vent DNA polymerase, using the oligonucleotides Pst-SLH (Mesnage *et al.*, 1999) and Nde-SLH (Table 1). The amplified fragment was inserted into pNEB193 digested with *Sma*I, giving rise to pSLH4045 (Fig. 1). The fragment was sequenced. A 2.67 kb fragment was then amplified by inverse PCR. Chromosomal DNA was digested with *Cla*I, for which there is one site in the fragment contained in pSLH4045. It was then self-ligated and used as a template for amplification with the divergent primers BT1 and BT2 (Fig. 1; Table 1). The sequence of the PCR product was determined directly. To sequence the 5' end of the gene, chromosomal DNA was digested with *Hind*III, for which there was a site in the second fragment amplified, 3' to the fragment from pSLH4045, and inverse PCR was carried out using BT15 and BT19 (Fig. 1; Table 1). A 1.2 kb product was obtained and directly sequenced. To check the DNA sequence encompassing all the cloning oligonucleotides, the 1.3 kb region from the *Mlu*I site to 270 bp 3' of BT19 was amplified from chromosomal DNA, using the oligonucleotides

Table 1. Oligonucleotide primers used for PCR and sequencing

Primer name	Sequence (5' → 3')
Nde-SLH	ATGTTTACTGTTAATGCGATAAATTGAGC
BT1	ACCCAGTGATCTTTTAAATCAGAGAA
BT2	GAAAAAGATGCTAACATCCTTAGTTG
BT15	CATCTTTAACTTCAGTACCGAACTTAACTTC
BT19	GCAGTTGTAGAAGGTTCAAAAGAAGCTTG
BT21	CGCGTTTTTCTATTTCATTTAAAATATTCTG
BT22	ACCGTTAATAACAACAGTAACTGCATCTTTGC
BT23	GTAATCGCTGGCACAATGACAGCAGCAATG
BT24	AGCGCTTTTGATTTGTTTCTCAATAAAGCTAAC
SLHup	AAATGGATCCGAGGTAAAACATTCCCAGACGTTCAA
SLHdown	TTAAAGCTTTTATTTTGTCCGAAGTGCATGTCAGTCTTAGC

Fig. 1. Schematic diagram of *Bac. thuringiensis* 4045 *slpA* region. (a) The *slpA* gene is represented by boxes corresponding to the various protein features. An arrow above indicates the direction of transcription of the gene identified from sequence data. The arrowheads at the ends of vertical dashed lines indicate the position and orientation of the oligonucleotides used for sequencing or cloning experiments described in this work. For clarity, they have been aligned to indicate the fragments obtained using the various pairs. The double bar indicates that the 1.2 kb DNA fragment obtained with primers BT15 and BT19 is not completely represented. (b) Schematic representation of the *Bac. thuringiensis* chromosomal fragments cloned in various vectors during this work. The names of the plasmids are on the left of the representations, those of the vectors used are in parentheses on the right. The only restriction sites indicated are those referred to in the text: C, *Clal*; H, *HindIII*; M, *Mlul*; S, *SapI*.

BT21 and BT22 (Fig. 1; Table 1), and the PCR product directly sequenced.

A $\Delta slpA$ strain was constructed as follows. A 2.57 kb fragment, starting with the tenth codon of *slpA* and ending 135 bp after the stop codon, was amplified from chromosomal DNA using BT23 and BT24 as primers (Fig. 1; Table 1). The fragment was inserted into pAT113 digested with *SmaI*, giving rise to pBT100 (Fig. 1). pBT100 was digested by *SapI* and a

spectinomycin cassette (Murphy, 1985), prepared by digesting pUC1318Spc with *HincII*, was inserted into it, giving rise to pBT101 (Fig. 1). HB101(pRK24) was transformed with pBT101 and the transformant used in mating experiments with *Bac. thuringiensis* NRRL 4045 to produce, by allelic exchange, SM4045.

Construction of a *Bac. thuringiensis* SLH-expression plasmid. The DNA fragment containing the three SLH motifs was

(a)

SlpA_Bt_4045	30	AGKTFPDPVQPGSWSAEYIDYLVAKKATECKPDGTFAPTEAIDRASAAKINAITLGLLEVKG---
SlpA_Bt_TDK	30	AGKSFPDPVQPGSWSAEYIDYLVAKKATECKPDGTFAPTEAIDRASAAKINAITLGLLEVKEG---
EA1_Ba_9131	30	AGKSFPDPVAGHWAEGSINYLVKGAITCKPDGTYGPTESIDRASAAYIFTKILNLPVDEN---
Olpa_B1_NM105	30	AGKSFPDPVAGHWAEDSINYLVKGAIVCKPDGTYGPTESIDRASAAYIFTKILNLPVDEN---
Sap_Ba_9131	30	AGKTFPDPVADHWCIDSYLVKGAIVCKNDKGMFEPGKELTRAAATMAAQIENLPIDKD---
SlpA_Bt_CTC	30	AGKSFPDPVADHWCIDSYLVKGAIVCKNDKGMFEPGKELTRAAATMAAQIENLPIDKD---
SlpA_Bf_OF4	34	ADAKFSDVSSSHWITDDINYLVEKGATQGYPDGTFKPNNSITRAFIAMVLANITGLVDVSEV---
SbsB_Bst_PV72	32	--ASFTDV--APQYKDAIDFLVSTGATKCKTETKFGVYDEITRLDAAVILARVUKLDVDNA---
SlpA_Bs_2362	31	--AQLNDFNKISGYAKEAVQSLVLAQVITQDANGNENPLKTIISRAAATFTNAELEAE----
SbpA_Bs_CCM2177	31	--AQVNDYNKISGYAKEAVQSLVLAQVITQDANGNENPLNITVTRAAAEFTKALELEAE----
HWP_Bb_HDP31	54	AEDTTTAPKMDAAMEKTVKRLEALGLVAGYNGDFGADKTIITRAEFATLVVRARGLLEQAKLAQ
MWP_Bb_47	57	EAAATTTAPKMDADMEKTVKRLEALGLVAGYNGEYGVDKTIITRAEFATLVVRARGLLEQAKLAQ
SlpA_Ct	27	DSFSEKEAEILRLGLYKGTSTETVYVNLLEGKLDQRGTGVVMLRLFGQEDDALETPMDEAAQT
SlpA_Bt_4045	91	AKPSFKDAQD--SWAAKYIAAVEQA--GVVQGDGTGNFNPNSQINRASMASMTVKAYKLEDKVSCG-----
SlpA_Bt_TDK	91	AKPSFKDAQD--SWAAKYIAAVEQA--GVVQGDGTGNFNPNSQINRASMATMTVKAYKLEDKVSCG-----
EA1_Ba_9131	91	AQPSFKDAKN--IWSSKYIAAVEKA--GVVKGDKGNFYEGKIDRASASMLVCAAYNLKDKVNGE-----
Olpa_B1_NM105	91	AQPSFKDAKN--IWSSKYIAAVEKA--GVVKGDKGNFYEGKIDRASASMLVCAAYNLKDKVNGE-----
Sap_Ba_9131	91	AKPSFADSQG--QWYTPFIAAVEKA--GVVKGDTGNG--FEPNGKIDRVSMASLLVAYKLETKVNCT-----
SlpA_Bt_CTC	91	AKPSFADSQG--QWYTPFIAAVEKA--GVVKGDTGNG--FEPNGKIDRVSMASLLVAYKLETKVNCT-----
SlpA_Bf_OF4	96	TTDRFSDVPATH--WANPYIAAIVDQTEGVLDGYENGTFRESNTITRQEMAKMVVAYDLLELVEG-----
SbsB_Bst_PV72	89	KDACFTDVPKDR--AKYVNAIVEA--GVVNGKAPGKFGAYDPTTRVEMAKIIANRYKLEKADD-----
SlpA_Bs_2362	89	GDVNFSDVKADA--WYYDAIAATVEN--GVVNGKAPGKFGAYDPTTRVEMAKIIANRYKLEKADD-----
SbpA_Bs_CCM2177	89	GDVNFSDVKKA--WYYNSTAAVVAN--GVVNGKAPGKFGAYDPTTRVEMAKIIANRYKLEKADD-----
HWP_Bb_HDP31	118	FNTTYTDVRSTD--WFAGFVNVAAGE--ELVKGFPDKSFKPONQVYAEAVTMTVRALGYEPPSVRCVWPNSMIS
MWP_Bb_47	121	FSNTYTDVKSTD--WFAGFVNVAAGE--ELVKGFPDKSFKPONQVYAEAVTMTVRALGYEPPSVRCVWPNSMIS
SlpA_Ct	91	LAARFKDAADIADWAQROVAYAVEK--GVVKGYPDGTFLPNADNGLAFCSLITQQGLYLGDFVF-----
SlpA_Bt_4045	153	LETFKFSDLKDH--WGEKDANILVALGIINCG---TGNGNEPDKSVTRAEAAKFIAKTDMQFGQK 210
SlpA_Bt_TDK	153	LETFKFSDLKDH--WGEKDANILVALDIINCG---TGNGNEPDKSVTRAEAAKFIAKTDMQFGQK 210
EA1_Ba_9131	153	LVTTFEDLDH--WGEKANILINLGISVCG---TGNGNEPDKSVTRAEAAKFIAKTDMQFGQK 210
Olpa_B1_NM105	153	LVTTFEDLDH--WGEKANILINLGISVCG---TGNGNEPDKSVTRAEAAKFIAKTDMQFGQK 210
Sap_Ba_9131	153	PATREKDLLETIN--WGEKANILVELGISVCG---TGNGNEPDKSVTRAEAAKFIAKTDMQFGQK 210
SlpA_Bt_CTC	153	PATREKDLLETIN--WGEKANILVELGISVCG---TGNGNEPDKSVTRAEAAKFIAKTDMQFGQK 210
SlpA_Bf_OF4	159	KDLFPFTDVSGL--WSTDYINILASNGVAAG---MTATTAPRGEVLRQAATAAFTHRAVEEERI 217
SbsB_Bst_PV72	147	VKLFPFTDVSGL--WSTDYINILASNGVAAG---MTATTAPRGEVLRQAATAAFTHRAVEEERI 217
SlpA_Bs_2362	149	DLSSEADASTVKKWAKSYLEIAVANGVIRKSEANGKTNLNPNAITTRQLFAVVFRTIENVDA-- 211
SbpA_Bs_CCM2177	149	SLSSEADASTVKKWAKSYLEIAVANGVIRKSEANGKTNLNPNAITTRQLFAVVFRTIENVDA-- 211
HWP_Bb_HDP31	188	KGSELNIAKGINNPNMQFAATIFKMLDNALRVKLMEQIEYGTDIRLNVTDETILTLYLKVTVR 251
MWP_Bb_47	191	KASELNIAKGINNPNMQFAATIFKMLDNALRVKLMEQIEYGTDIRLNVTDETILTLYLKVTVR 254
SlpA_Ct	154	DEAAVKLQFEGGLTAEQAEAFNNKNGINRDS--MVGIAESALQAVYKATCKTVIEVLVENGNV-- 214

Fig. 2. For legend see facing page.

amplified using the oligonucleotides SLHup and SLHdown and inserted into pNEB193 digested with *Sma*I to give pNSLH100 (Fig. 1; Table 1). pQSLH100 was constructed by digesting pNSLH100 with *Bam*HI and *Hind*III and inserting the resulting fragment into pQE30 digested similarly (Fig. 1). The plasmid was checked by sequencing.

Protein analysis. *Bac. thuringiensis* bacteria were grown overnight in BHI and pelleted by centrifugation. Pellets were washed and resuspended in 1/10 the initial volume of 2 M guanidinium hydrochloride (pH 2.5), as described by Luckevich & Beveridge (1989) to extract specifically the cell-surface-anchored proteins. The samples were then dialysed against Tris/HCl (pH 8.0), resuspended in Laemmli buffer (Laemmli, 1970), boiled and 20 µl was loaded onto 10% polyacrylamide gel and subjected to electrophoresis. The gels were fixed and stained with Coomassie brilliant blue.

In vitro interaction of the purified SLH domain with *Bac. thuringiensis* and *Bac. anthracis* cell walls. The His-tagged SLH domain of *Bac. thuringiensis* was purified according to Qiagen's recommendations, as described previously (Mesnage

et al., 1999), with elution at an imidazole concentration of 50 mM. The cell walls of both *Bac. thuringiensis* NRRL 4045 and *Bac. anthracis* SM11 strains were prepared as described previously (Mesnage *et al.*, 1999). The interaction of the *Bac. thuringiensis* SLH domain with the cell walls was also studied as described previously (Mesnage *et al.*, 1999).

Extraction of polysaccharide, carbohydrate assay and determination of monosaccharide composition. The total polysaccharide fraction was extracted from cell walls as described by Ekwunife *et al.* (1991). Monosaccharide composition was determined by GC on polyol acetate, as described by Sawadeker *et al.* (1967).

RESULTS AND DISCUSSION

Isolation and sequence analysis of the gene encoding the component of the *Bac. thuringiensis* subsp. *galleriae* NRRL 4045 S-layer

The first seven amino acid residues of *Bac. thuringiensis* 4045 mature S-layer protein are identical to the cor-

(b)

SlpA_Bt_4045	211	-----AAKVESIKKBNNAKEHEVKGFEVVKDVTAA--FAVVEGSKEDIERVELSKDKKSATITLKN-----ALANKQAVVAHVVKDKSVHGR--IPK
Slp_Bt_TDK	211	-----AAKVESIKKBNNAKEHEVKGFEVVKDVTAA--FAVVEGSKEDIERVELSKDKKSATITLKN-----ALANKQAVVAHVVKDKSVHGR--IPK
Sap_Ba_9131	211	-----AAKVESAKAVTTQKHEVKGFEVVKDVTAA--FAVVEGSKEDIERVELSKDKKSATITLKN-----ALANKQAVVAHVVKDKSVHGR--IPK
Slp_Bt_CTC	212	-----AAKVESAKAVTTQKHEVKGFEVVKDVTAA--FAVVEGSKEDIERVELSKDKKSATITLKN-----ALANKQAVVAHVVKDKSVHGR--IPK
EA1_Ba_9131	211	DNAQAVYTVVKVSEPTKLTTLTGTDKLSADVTLEEDKAAVAEASTDGTSAVVTLGKGVAPNKDLTVRVKNQSPVTRFVYEVVKLAVERKTFDDDRAGQ
Olpa_B1_NM105	211	ENSDAKVTNVAATEPTQTLTLTGTDKLSADVTLEEDKAAVAEASTDGTSAVVTLGKGVAPNKDLTVRVKNQSPVTRFVYEVVKLAVERKTFDDDRAGQ
SlpA_Bt_4045	297	AEVIFPNDVAPTVSTSTPDGNGVWV--FSBKSKDAITVINGKEPTATPENTTLTKADVASVVKNGEPFNVIVTCAKIVNTMEYEGRATYKVE
Slp_Bt_TDK	301	AEVIFPNDVAPTVSTSTPDGNGVWV--FSBKSKDAITVINGKEPTATPENTTLTKADVASVVKNGEPFNVIVTCAKIVNTMEYEGRATYKVE
Sap_Ba_9131	298	EAKTIEMADQTVVADEPTALQF--TVKDENGTEVSPET--BFVTFPAEKNINAGEITLAKCTSTTVKAVVKKDKGVVAESKEVKSSEGAASISNMTVAE
Slp_Bt_CTC	299	EAKTIEMADQTVVADEPTALQF--TVKDENGTEVSPET--BFVTFPAEKNINAGEITLAKCTSTTVKAVVKKDKGVVAESKEVKSSEGAASISNMTVAE
EA1_Ba_9131	311	AAAFKLNDKGNADVEYINLANHDKVWV--ANNIDGSPANIEFEGGTAISTTGKLAAGCKGCDYKVEVQVTKKCGILVSNCTGILTVKNLDTPASAIKNNVFAL
Olpa_B1_NM105	311	AAAFKLNDKGNADVEYINLANHDKVWV--ANNIDGSPANIEFEGGTAISTTGKLAAGCKGCDYKVEVQVTKKCGILVSNCTGILTVKNLDTPASAIKNNVFAL
SlpA_Bt_4045	397	KDVTAPEVKDIDVVKSLVIG--YATIEVTFSEELSAAG--KVIVKKGDKVLEGATIAEDDITKALVTVPAALTKETAAK--VEFGYKDAANNVGNKVT
Slp_Bt_TDK	399	KDVTAPEVKDIDVVKSLVIG--YATIEVTFSEELSAAG--KVIVKKGDKVLEGATIAEDDITKALVTVPAALTKETAAK--VEFGYKDAANNVGNKVT
Sap_Ba_9131	398	QNKADPTSKDFRNNKVE--GDNAYVQVBLKQDFN--AVTTGKVEYESINAEVAVDKATGKVTIVSACAKAPVKVTVKDSKGKELVSKTVEIEAFQKAM
Slp_Bt_CTC	399	QNKADPTSKDFRNNKVE--GDNAYVQVBLKQDFN--AVTTGKVEYESINAEVAVDKATGKVTIVSACAKAPVKVTVKDSKGKELVSKTVEIEAFQKAM
EA1_Ba_9131	411	ADNDGVNNGSKLSGKIFALNNQNVVGEKASINKLNATHAGEDVVDPGSISIKSSNHCHISVNNYITAEAGAEITLTVKGVDTKDKVFKVTTDSR
Olpa_B1_NM105	411	ADNDGVNNGSKLSGKIFALNNQNVVGEKASINKLNATHAGEDVVDPGSISIKSSNHCHISVNNYITAEAGAEITLTVKGVDTKDKVFKVTTDSR
SlpA_Bt_4045	493	KEVKVVKDVAAPVVKVADENKAAFTFDKRVTAQEGKLVNLTSTSEVTKEVAVASVEDNKKATLTPDEKGNVKAATKGEV-----
Slp_Bt_TDK	498	KEVKVVKDVAAPVVKVADENKAAFTFDKRVTAQEGKLVNLTSTSEVTKEVAVASVEDNKKATLTPDEKGNVKAATKGEV-----
Sap_Ba_9131	495	KEVKVVKDVAAPVVKVADENKAAFTFDKRVTAQEGKLVNLTSTSEVTKEVAVASVEDNKKATLTPDEKGNVKAATKGEV-----
Slp_Bt_CTC	496	KEVKVVKDVAAPVVKVADENKAAFTFDKRVTAQEGKLVNLTSTSEVTKEVAVASVEDNKKATLTPDEKGNVKAATKGEV-----
EA1_Ba_9131	511	KLVSVKANPDKLVVQNTKEVTFVVTDOYGEFFGANTAAIKVLPKGVVVEGGLDVVTTDSGSGTITIGVTGNDVGEETVHFQ--NGNGATLGSLYV
Olpa_B1_NM105	510	KLVSVKANPDKLVVQNTKEVTFVVTDOYGEFFGANTAAIKVLPKGVVVEGGLDVVTTDSGSGTITIGVTGNDVGEETVHFQ--NGNGATLGSLYV
SlpA_Bt_4045	579	---KDTAGNESAPTKVVKVVEKKEGKKDEVAAP-----ATKVERVADSKTKFTTDFKEVKGGQAGSASNNVNYILAGAKPEPTLIVNAD
Slp_Bt_TDK	584	---KDTAGNESAPTKVVKVVEKKEGKKDEVAAP-----ATKVERVADSKTKFTTDFKEVKGGQAGSASNNVNYILAGAKPEPTLIVNAD
Sap_Ba_9131	580	---EKBAKATLLELKAAPGAFSKFEVVGLEKLDK-----VYTEENKKNEMVSVLPVDANGLVLEKKEBATLKVITIDRDKVVDATSGQAVN
Slp_Bt_CTC	581	---EKBAKATLLELKAAPGAFSKFEVVGLEKLDK-----VYTEENKKNEMVSVLPVDANGLVLEKKEBATLKVITIDRDKVVDATSGQAVN
EA1_Ba_9131	609	NVTEGNVAFKN--FELVSKVQYQYQSPDTKL--LVNSTTVEYQLSKYTSIRVSDPENIEGYEVSKNLAVADAKIVSNKVVVTKRTPGKVDIHLTKNGAT
Olpa_B1_NM105	608	NVTEGNVAFKN--FELVSKVQYQYQSPDTKL--LVNSTTVEYQLSKYTSIRVSDPENIEGYEVSKNLAVADAKIVSNKVVVTKRTPGKVDIHLTKNGAT
SlpA_Bt_4045	666	QSVTIEETPTTFPERSETVKFTVANVANKGVKMGNTNLLVNVDTKAPPEKSAKITKVDAAKEHTTFS--EAVNVDATFVVDINGVLTATKDETAE
Slp_Bt_TDK	669	QSVTIEETPTTFPERSETVKFTVANVANKGVKMGNTNLLVNVDTKAPPEKSAKITKVDAAKEHTTFS--EAVNVDATFVVDINGVLTATKDETAE
Sap_Ba_9131	667	NNS-VTVVCGAKAG--ETVKVTVVLDG-----KLITTSFVKVDAPAKKLAADFSTSLNEVAQGE--LKTALLNLSVDCGPTTGGATVTKPV
Slp_Bt_CTC	668	NNS-VTVVCGAKAG--ETVKVTVVLDG-----KLITTSFVKVDAPAKKLAADFSTSLNEVAQGE--LKTALLNLSVDCGPTTGGATVTKPV
EA1_Ba_9131	707	AGKATVETVOETIANK--SVNFKPVCTENFVEKKKNIGTLEDEKSNLDDIVKGINLTKETQKVRVKSAGECKLYLDRNGDAVFNAEDVKLGDDTVS
Olpa_B1_NM105	706	AGKATVETVOETIANK--SVNFKPVCTENFVEKKKNIGTLEDEKSNLDDIVKGINLTKETQKVRVKSAGECKLYLDRNGDAVFNAEDVKLGDDTVS
SlpA_Bt_4045	765	ASKDVVLKVT-----PADVNLATCTVTVKAKEVECKVNLNATSTINKKLVAFKPVATATE--
Slp_Bt_TDK	767	ASKDVVLKVT-----PADVNLATCTVTVKAKEVECKVNLNATSTINKKLVAFKPVATATE--
Sap_Ba_9131	758	SADTNVVAEN-----GTVGAKGATSIYVKNLTVKDGKECKVFDRAVCVAVSKEAKPATK--
Slp_Bt_CTC	760	SADTNVVAEN-----GTVGAKGATSIYVKNLTVKDGKECKVFDRAVCVAVSKEAKPATK--
EA1_Ba_9131	805	QTSFSAALPN-----FKADYDTITTKYTDKCTLVKFKLKDQVITSEIGSQAVHVNVLNNPNL
Olpa_B1_NM105	804	QTSFSAALPN-----FKADYDTITTKYTDKCTLVKFKLKDQVITSEIGSQAVHVNVLNNPNL

Fig. 2. Similarity between the sequences of SlpA from *Bac. thuringiensis* 4045 and other *Bac. cereus* S-layer proteins. (a) SLH anchoring motifs of *Bacillaceae* S-layer proteins: *Bac. thuringiensis* subsp. *galleriae* NRRL 4045 (SlpA_Bt_4045; accession no. AJ249446), *Bac. thuringiensis* subsp. *israelensis* 4Q2 (Slp_Bt_TDK; CAA44000), *Bac. thuringiensis* subsp. *finitimus* CTC (Slp_Bt_CTC; CAA09981), *Bac. anthracis* 9131 (EA1_Ba_9131; CAA68063), *Bac. anthracis* 9131 (Sap_Ba_9131; CAA85408), *Bac. licheniformis* NM105 (Olpa_B1_NM105; AAC44405), *Bacillus firmus* OF4 (Slp_Bf_OF4; AF242295), *Bac. stearothersmophilus* PV72 (SbsB_Bst_PV72; CAA66724), *Bacillus sphaericus* 2362 (Slp_Bs_2362; P38537), *Bac. sphaericus* CCM2177 (SbpA_Bs_CCM2177; AF211170), *Bre. brevis* 47 (MWP_Bb_47; A28555), *Bre. brevis* HDP31 (HWP_Bb_HDP31; A35129) and *Clostridium thermocellum* NCIB 10682 (SlpA_Ct; AAC33404). A gap has been left between the first six sequences and the others to indicate the lower level of similarity between the sequences of the SLH motifs of the *Bac. cereus* group, to which *Bac. licheniformis* is linked, and the others. (b) Putative crystallization domains of *Bac. cereus* and *Bac. licheniformis* S-layer proteins. A gap has been left between the first four sequences and those of EA1 and OlpA to indicate the lower level of similarity between the last two sequences and the others. The order is that generated using the CLUSTAL method. Amino acid residues identical or conserved in at least half the sequences are shown on a black or grey background, respectively.

responding residues of Sap (Etienne-Toumelin *et al.*, 1995; Luckevich & Beveridge, 1989). These residues are included in the SLH domain and in fact are, except for one conservative change, also identical to those of EA1 (Mesnage *et al.*, 1997). SLH motifs share a high level of

sequence conservation. These data suggest that the *Bac. thuringiensis* S-layer protein starts with an SLH domain. To amplify the SLH motifs, we designed two oligonucleotides, Pst-SLH and Nde-SLH (Mesnage *et al.*, 1999; Table 1). An initial fragment was thus amplified,

cloned (pSLH4045) and sequenced. Like Sap and EA1, and other Gram-positive S-layer proteins, the *Bac. thuringiensis* protein harbours three SLH motifs (Mesnage *et al.*, 1997; and for review, see Engelhardt & Peters, 1998). In two steps of inverse PCR, the DNA fragment overlapping the complete ORF was amplified and sequenced. The corresponding gene was named *slpA*. Sequencing identified an ORF, encoding a protein with a predicted molecular mass of 87.2 kDa. Analysis of the deduced amino acid sequence showed that there was a 29 aa signal peptide followed by three SLH motifs. The mature protein thus starts, as predicted, with AGKTFPDV (Luckevich & Beveridge, 1989) and has a predicted molecular mass of 84.4 kDa, which is similar but not identical to the 91.4 kDa published (Luckevich & Beveridge, 1989). A difference between the predicted molecular mass and that deduced from electrophoretic migration has already been observed with Sap and EA1 (Etienne-Toumelin *et al.*, 1995; Mesnage *et al.*, 1997). Furthermore, in *Bac. anthracis*, MS confirmed the molecular mass deduced from the sequence for both Sap and EA1 proteins (O. Barzù, personal communication). A pI of 5.33 was calculated from the complete sequence and this is close to the value of 5.0 obtained by isoelectric focusing (Luckevich & Beveridge, 1989).

A BLASTP (v. 2.0.14) search was carried out with the complete translation product (SlpA) and with each of the putative domains (i.e. the SLH motifs alone and the protein sequence with these motifs deleted) (Altschul *et al.*, 1997). If the complete protein was used, Sap and EA1 were the second and fourth most similar proteins, with a Poisson probability or *E* value of 3×10^{-74} and 4×10^{-73} , respectively. If the SLH domain was used alone EA1 and Sap had the second and third most similar SLH domains respectively (*E* values of 5×10^{-66} and 3×10^{-63} , respectively). These domains were probably responsible for the high scores obtained with the complete proteins. The CLUSTAL comparison between the SLH domains of S-layer proteins from *Bacillus* species shows that there is a high level of sequence conservation for the anchoring domain (Fig. 2a). Each motif more closely resembles the equivalent motifs of the other proteins than other motifs of the same protein. If only the putative crystallization domains were compared, protein similarity was observed only with Sap, with an *E* value of 9×10^{-9} . CLUSTAL analysis was then carried out with the putative crystallization domain of known *Bac. cereus* S-layer proteins and OlpA from *Bacillus licheniformis*, which closely resembles EA1 (Fig. 2b) (Mesnage *et al.*, 1997; Zhu *et al.*, 1996). The alignment of these domains suggests that the sequences of these proteins, within the *Bac. cereus* group, are more conserved than any two randomly chosen S-layer proteins (Fig. 2b and data not shown). If OlpA and the accompanying *Bac. anthracis* EA1 sequences were omitted from the comparison, the sequences appeared even more conserved (Fig. 2b, upper cluster). This suggests that the other four proteins compared may have had a common ancestor, closer to that of Sap than EA1. The common sequences conserved in Sap, CTC and SsbC

Fig. 3. Analysis of *Bac. thuringiensis* 4045 and SM4045 surface proteins. SDS-PAGE of cell-wall fractions of parental (lane 1) and $\Delta slpA$ mutant (lane 2) strains. The positions of molecular mass markers are indicated on the left.

from *Bac. stearothermophilus*, as described by Jarosch *et al.* (2000), are not very well conserved in our alignment, except for the charged residues and lysines in particular, which may reflect the high proportion of these residues in S-layer proteins. Thus, as is often discussed, the sequences of S-layer proteins, even from related organisms (e.g. *Bacillus*) display little or no identity (Sára & Sleytr, 2000). To test whether the sequenced gene was that encoding the S-layer protein described by Luckevich & Beveridge (1989), we inactivated the gene and analysed the proteins present at the surface of the mutant strain.

Construction and characterization of an *slpA*-disrupted mutant

The *slpA* gene was sequenced directly from PCR products and not cloned. To construct a disruption, we had to clone a fragment overlapping the sequence. This was done by PCR amplification and insertion of the corresponding fragment into a conjugative plasmid (see Methods). A disrupted copy was then constructed by inserting a resistance cassette in the middle of the sequence. By mating *E. coli* harbouring the construct and *Bac. thuringiensis* 4045, we obtained a recombinant strain, SM4045, which we then studied. The integration of the plasmid copy at the chromosomal locus had, as expected, occurred by a double crossover event, giving rise to an allelic exchange.

Luckevich & Beveridge (1989) described a specific extraction procedure for the S-layer protein of *Bac. thuringiensis* 4045. This method, which involves resuspending the cells in guanidinium hydrochloride at

Fig. 4. *In vitro* interaction of the SLH domains of SlpA (a and b) and Sap (c) with purified *Bac. thuringiensis* 4045 (a and c) or *Bac. anthracis* SM11 (b) cell wall. Each SLH domain was incubated in the absence (1) or presence (2) of purified cell wall. In (3), cell walls were treated with hydrofluoric acid, thus removing the associated polysaccharide, before incubation with the SLH domains. Lanes: S, soluble fraction (unbound proteins); I, insoluble fraction (bound or self-aggregated proteins).

low pH as a chaotropic agent, was used on the parental strain and the disrupted SM4045 mutant (Fig. 3). The absence of the 91 kDa protein in the cell-wall fraction of SM4045 confirmed the deletion of the structural gene encoding the *Bac. thuringiensis* 4045 S-layer protein. It also suggested that no other high-molecular-mass protein was abundant on the surface of *Bac. thuringiensis* 4045. This contrasts with the situation in other bacteria, including various *Bacillus* species such as *Bac. anthracis* or *Bac. stearothermophilus*, which synthesize two S-layer components. In *Bac. anthracis*, both proteins are found on the cell surface of the parental strain and in *Bac. stearothermophilus*, one protein is replaced by another if the medium is changed. Similarly, in other bacteria, a second S-layer protein appears if the gene encoding the S-layer of the parental strain is disrupted (Sára & Sleytr, 2000). This striking difference between *Bac. anthracis* and *Bac. thuringiensis* 4045 suggests that the S-layer may provide a selective advantage in certain environmental conditions. It is therefore possible that differences in the S-layer reflect different ecological niches or different requirements for the presence of this structure. As the S-layer constitutes 5–10% of total cell protein, its synthesis presumably requires an energy input from the bacteria.

Analysis of the SlpA SLH-motifs as an anchoring domain

Sequencing indicated the presence of three SLH motifs extremely similar to those of *Bac. anthracis* S-layer proteins. We have previously shown that the SLH motifs of *Bac. anthracis* are able to bind purified *Bac. anthracis* cell wall unless the wall is treated to deplete it of the associated pyruvate-containing polysaccharide (Mesnage *et al.*, 1999, 2000). Genetic and biochemical experiments also suggested that similar binding should occur for SLH-containing proteins from various bacteria including *Bac. thuringiensis* 4045 (Mesnage *et al.*, 2000). We tested this conclusion directly. The SlpA-SLH

domain was overproduced in *E. coli* and its capacity for binding to a *Bac. thuringiensis* cell-wall preparation was analysed (Fig. 4). The SLH domain specifically bound the *Bac. thuringiensis* cell wall unless the associated polysaccharide was removed by hydrofluoric acid treatment (Fig. 4a). There was also a perfect cross-reaction between *Bac. thuringiensis* and *Bac. anthracis* SLH domains and cell walls. Indeed, the SlpA-SLH domain also bound specifically to the *Bac. anthracis* cell wall (Fig. 4b) and the Sap-SLH domain equally bound to *Bac. thuringiensis* cell wall (Fig. 4c). In *Bac. anthracis*, the peptidoglycan-associated polysaccharide binding the SLH domain is pyruvylated and contains galactose, N-acetylglucosamine and N-acetylmannosamine in a molar ratio of 10:3:1. We have previously shown that the cell wall of *Bac. thuringiensis* 4045 also contains pyruvate (Mesnage *et al.*, 2000). PCR co-amplification of an *slh* sequence and the upstream gene, *csaB*, the product of which is involved in pyruvylation of the peptidoglycan-associated polysaccharide, gave a positive signal with *Bac. thuringiensis* 4045 chromosomal DNA (Mesnage *et al.*, 2000). We determined the composition of the peptidoglycan-associated polysaccharides from *Bac. thuringiensis*. Like that of *Bac. anthracis*, the polysaccharides from *Bac. thuringiensis* contained galactose, N-acetylglucosamine and N-acetylmannosamine, but they also contained glucose and N-acetylgalactosamine in the molar ratio 6:10:3:5:6:5. Further analyses are therefore required to determine which carbohydrate moiety or moieties are pyruvylated in each of these bacteria, and whether the SLH domains bind to the same ligand.

Concluding remarks

We devised and successfully tested a simple method suitable for the cloning and disruption of most, if not all, S-layer genes from the *Bac. cereus* group. Previous analyses showed that S-layer proteins within the *Bac. cereus* group are likely to harbour SLH domains and that these SLH domains interact non-covalently with pyruvylated wall-associated polysaccharides (Mesnage *et al.*, 2000). Based on these observations, we designed oligonucleotides to amplify DNA fragments encoding the SLH domain from S-layer proteins of bacteria belonging to the *Bac. cereus* group. The high level of conservation of the SLH sequences, highlighted in Fig. 2(a), indeed confirms that SLH domain sequences of S-layer proteins are highly conserved within the *Bac. cereus* group, allowing cloning of the corresponding DNA fragment by PCR amplification with the described oligonucleotides. A one- to two-step inverse PCR is sufficient to isolate the rest of the S-layer gene sequence (as in Fig. 1, using *Clai* and BT1 and BT2, and then *HindIII* and BT15 and BT19). The gene, or part of it, is then amplified and inserted into a suicide conjugative vector such as pAT113, which has a broad host range (as in Fig. 1, with BT23 and BT24) (Trieu-Cuot *et al.*, 1991). It is not advisable to clone the gene with its regulatory region as this could lead to problems and in many cases

has proved impossible (Lemaire *et al.*, 1998 and references therein). The gene is then disrupted by insertion of a selectable marker, such as the spectinomycin resistance cassette inserted into the *SapI* site in this study, and the copy harbouring the resistance cassette is integrated into the *Bac. cereus* chromosome at the original locus. The disruption resulting from an allelic exchange due to a double crossover event makes reversion impossible, enabling *in vivo* studies. Such approaches will be of great value for the molecular and functional characterization of *Bac. cereus* S-layers, facilitating the comparison of genetically engineered S-layer-deficient isogenic strains. Using degenerate oligonucleotides, taking into account codon usage, this technique could also be applied to many other bacterial species in which genetic transfer is possible.

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