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The Bacterial Surface Layer Provides Protection against Antimicrobial Peptides

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This report describes a previously unrecognized role for bacterial surface layers as barriers that confer protection against antimicrobial peptides. As antimicrobial peptides exist in natural environments, S-layers may provide a bacterial survival mechanism that has been selected for through evolution.

Proteinaceous paracrystalline surface layers (S-layers) cover the cell walls of many members of the *Bacteria* and *Archaea* and may be one of the oldest forms of bacterial cell envelopes (2). In fact, they are present in a broad range of bacteria, including environmental species, pathogens, and bioterrorism-related agents (e.g., *Bacillus anthracis*, *Bacillus cereus*, *Campylobacter fetus*, *Campylobacter rectus*, *Aeromonas salmonicida*, and *Bacillus sphaericus*) (4, 12, 14, 16). S-layers are mostly composed of protein or glycoprotein monomers that can play roles as virulence factors, adhesion receptors, or protective barriers against immune system attacks (2). Some bacterial S-layers have been shown to protect against predation by *Bdellovibrio* species (9). More specifically, the S-layer of *Caulobacter crescentus* has been shown to offer protection against the predator bacterium *Bdellovibrio exovorus* (10). In most *Archaea*, the S-layer is the only cell wall structure and is thought to be important for mechanical stabilization (2). Nonetheless, the function of most S-layers remains largely unknown. It has been estimated that 7 to 30% of protein synthesis is dedicated to S-layer production, suggesting that this structure has an essential cellular role (11). Here we considered the possibility that the preservation of the S-layer through evolution might reflect a role in bacterial survival. Specifically, we hypothesized that the main function of the S-layer is to protect bacteria against exogenous stresses, particularly those derived from exposure to antibiotic molecules produced by competitor bacteria or by other organisms present in the environment (fungi, plants, etc.). Further, we argued that, although it is generally thought that S-layers allow free passage of small molecules (19), cellular protection could still be achieved through charge interactions. The majority of bacterial S-layers found in nature are acidic, with pIs of around 4 (7, 16, 25). S-layer-related protection may therefore be especially important against cationic antimicrobial peptides.

For this study, we focused on the S-layer of the environmental Gram-negative bacterium *Caulobacter crescentus* (21). This S-layer is composed of about 40,000 monomers of a 98-kDa protein called RsaA that self-assembles as a hexagonally arranged structure on the cell surface (21). By negative staining and electron microscopy it was observed that the S-layer forms pores with a diameter of 2.5 to 3.5 nm and a thickness of ~7 nm (21). Based on these porosity estimates, all peptides used in this study should have been able to traverse the S-layer. To test the potential role of the S-layer in protection against antibiotics, an S-layer-negative strain, JS4026 [closely related to strain CB15 (18); S-layer negative and holdfast negative, with plasmid replication genes (*repBAC*) inserted into

xylX (11)], and an S-layer-positive strain [JS4026 complemented with plasmid p4B:*rsaA600*, a plasmid containing *rsaA* under the control of the native *rsaA* promoter (11)] of *C. crescentus* were used. These strains were grown in PYE medium (21) at 30°C both in test tubes and in 96-well plates and were challenged with increasing levels of antimicrobial agents, including the human peptide LL-37 and synthetic peptides IDR-1018 and 1037 (1). Significant protection against antimicrobial peptides but not other antibiotics was observed in cells containing the S-layer. These initial results highlighted an antimicrobial peptide-specific intrinsic resistance mediated by the S-layer of *C. crescentus*, as no protection was observed when the cells were exposed to nonpeptide antibiotics such as ceftazidime, ciprofloxacin, and tetracycline (data not shown). To confirm this S-layer-mediated resistance against antimicrobial peptides, we treated two additional isogenic strains, the S-layer-positive strain NA1000 [variant of CB15, holdfast negative; (5)] and the S-layer-negative strain JS1013 [NA1000 with a 3530B frameshift mutation; holdfast negative (6)], with 9 additional peptides. Interestingly, MIC assays (24) revealed that, in most cases, the S-layer was able to increase resistance to antimicrobial peptides at least 2-fold (Table 1). Similarly, optical density readings from 96-well plates, in which both strains were grown in PYE at 30°C for 24 h and were treated with increasing peptide concentrations, consistently showed protection, although the extent of protection varied according to the peptide used (data not shown). The greatest level of protection was obtained against the highly cationic peptide LP-1 (Table 1). In contrast, no significant protection was observed when the negatively charged lipopeptide daptomycin was used (Table 1). Peptide size, on the other hand, appeared to have only a modest effect (Table 1). It is well known that low to moderate concentrations of antimicrobial peptides are produced by virtually all living organisms, including bacteria, fungi, plants, and animals (8, 17). Hence, the results of this first set of experiments were in keeping with our hypothesis that the S-layer is involved in bacterial survival.

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TABLE 1 Peptides in decreasing order of net charge^a

Peptide	Amino acid sequence ^b	Mol wt (g/mol)	Charge	MIC ($\mu\text{g/ml}$) for:	
				S-layer negative JS1013	S-layer positive NA1000
LP-1	RKRKRKR(K-myristoyl)	1,512.6	+9	2	8
CALL	KWKLFKKIFKRIVQRIKDFLR	2,791.5	+8	8	16
BMAP-28	GGLRSLGRKILRAWKKYGPIIVPIIRIG	3,130.9	+7	4	8
CRAMP	ISRLAGLLRKGGKIGKIGKIKKIGKIKNFFQKLVQPPE	4,291.2	+7	2	4
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	4,391.3	+6	2	4
1037	KRFRIRVRV	1,229.5	+5	2	4
Bac2A	RLARIVVIRVAR	1,421.8	+4	4	8
1018	VRLIVAVRIWRR	1,536.9	+4	4	8
W3	VRWIVAVRIWRR	1,610.0	+4	4	8
Pleurocidin	GWGSFFKKAHVGVKGVKAAALHTHYL	2,711.2	+4	1	2
Indolicidin	ILPWKWPWPWRR	1,907.3	+3	4	8
Daptomycin	<i>n</i> -Decanoyl-WN ^D DTGODA ^D DGS ^D TEK	1,619.7	-3	8	8

^a Bacteria were grown for 24 h at 30°C in PYE medium, which includes 0.01% CaCl₂, as daptomycin is Ca²⁺ dependent. MICs were determined as previously described (20). Data correspond to at least three independent experiments, each consisting of eight replicates for every condition tested.

^b A superscript D signifies the D isomer. O, ornithine; K, kynurenine.

Next, we performed killing assays in order to obtain further understanding of the level of protection offered by the S-layer. Previous studies have shown that killing assays are more sensitive than optical density readings and MIC measurements (13). The S-layer-positive strain NA1000 and S-layer-deficient strain JS1013 were grown in PYE for 24 h at 30°C with shaking and then treated

with antimicrobial peptide LP-1 and the commonly studied cationic peptide LL-37 (Table 1). Killing curves were performed using PYE agar plates for 4 h as previously described (20). As predicted, NA1000 exhibited increased resistance to antimicrobial peptides compared to the strain lacking the S-layer (Fig. 1). Indeed, NA1000 exhibited an $\sim 10,000$ -fold increase in resistance to

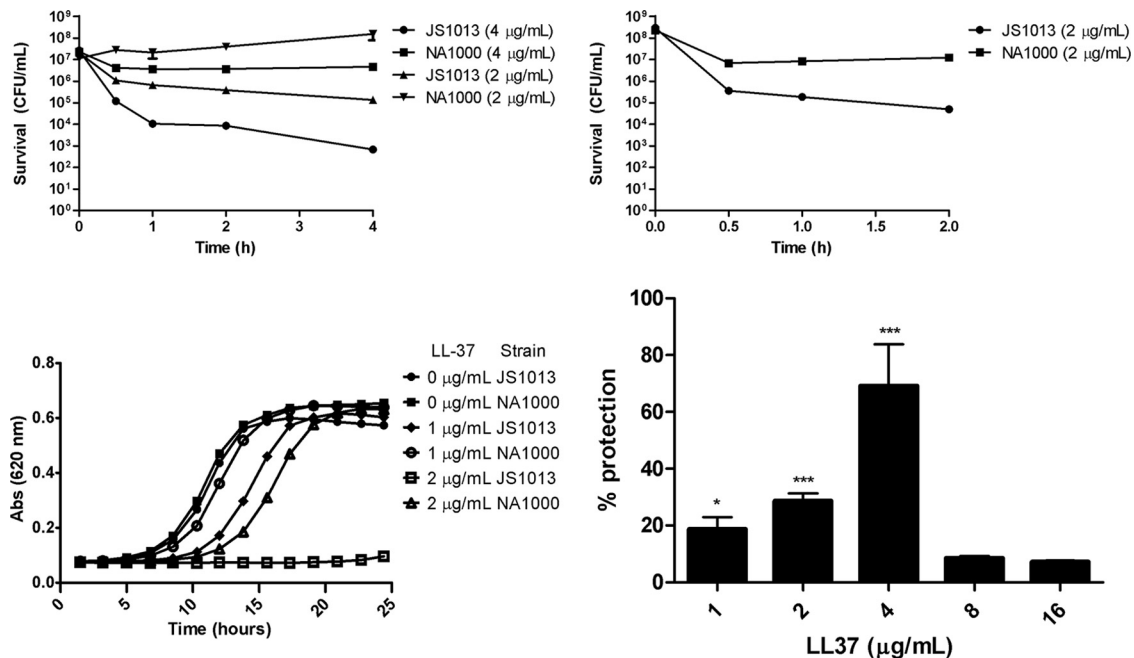


FIG 1 The *C. crescentus* S-layer provides protection against antimicrobial peptides LP-1 (top left) and LL-37 (top right) in killing assays and LL-37 in growth curves (bottom left). Strains used were NA1000 (S-layer positive) and JS1013 (S-layer negative). (Bottom right) Biofilms of an S-layer-positive strain were more resistant to the antimicrobial peptide LL-37 than their S-layer-negative counterparts. Optical densities at 600 nm (OD₆₀₀) of biofilm, after crystal violet staining, were measured for peptide-treated samples of both S-layer-positive (CB15) and S-layer-negative (CB15 Δ RsaA) strains, and the OD₆₀₀ values were converted into percentages by dividing the values obtained by the averages of their respective negative controls (i.e., no peptide treatment). The level (percent) of protection provided by the S-layer was calculated by subtracting the percentages for the S-layer-negative strain from those for the S-layer-positive strain at each peptide concentration. All experiments were done at least three times. Data in the bottom panels correspond to one experiment consisting of 8 replicates with similar results being obtained in at least 3 independent experiments. For the bottom right panel, *t* tests were performed to assess significance levels at each peptide concentration (*, $P < 0.05$; ***, $P < 0.001$).

different concentrations of LP-1, compared to JS1013 (Fig. 1). Likewise, the S-layer offered ~100-fold protection against LL-37 (Fig. 1). These results further highlight the protective effect of the S-layer. Growth curves were also performed to compare treated and untreated S-layer-positive and S-layer-negative strains. Strains NA1000 and JS1013 were grown at 30°C with shaking in the absence and presence of LL-37. Effects were monitored with a TECAN Spectrofluor Plus spectrometer by determining the absorbance at 620 nm every 20 min for 24 h. At 2 µg/ml LL-37, NA1000 grew to levels comparable to those of the untreated sample (Fig. 1). In contrast, JS1013 was completely killed (Fig. 1). Also, the 3530B frameshift mutation introduced into the S-layer-negative strain JS1013 did not affect growth of this strain compared to wild-type *C. crescentus* NA1000 (Fig. 1).

C. crescentus has been shown to be capable of forming dense biofilms when grown axenically (3, 22). Further, we questioned whether the S-layer could protect against antimicrobial peptides when the organism is grown in biofilms, as bacteria in nature often grow associated to surfaces (1, 15). To address this point, we used the biofilm-forming strains CB15 [ATCC 19089, wild-type strain, S-layer positive, holdfast positive (18)] and CB15ΔRsaA [CB15 with complete RsaA knockout, S-layer negative, holdfast positive (23)] in which the holdfast (i.e., necessary for biofilm formation) was not knocked out. Both strains were grown under biofilm conditions using 96-well plates, and biofilm formation was quantified by staining adherent cells with crystal violet and recording absorbance levels at 595 nm, as previously described (1). Interestingly, *C. crescentus* biofilms exhibited increased resistance to peptide LL-37 and were able to grow at 2 and 4 µg/ml of peptide, which inhibited the growth of S-layer-negative planktonic cells (Fig. 1). This allowed us to demonstrate that biofilms formed by S-layer-positive CB15 exhibited increased resistance to LL-37, compared to S-layer-negative CB15ΔRsaA (Fig. 1; shown as percent protection against biofilm inhibition). Similar results were also obtained with LP-1 (data not shown). In summary, these experiments provided additional support for the involvement of the S-layer in bacterial survival. In environments containing antimicrobial peptides, S-layer-producing bacteria had a clear advantage over bacteria lacking the S-layer.

To obtain some mechanistic insights and determine whether the S-layer protein itself was sufficient to explain the observed protection, RsaA protein was isolated, and increasing concentrations were added to JS1013 cultures treated with 2 µg/ml LL-37. Virtually 100% survival was observed when as little as 62.5 µg/ml of RsaA protein was used to supplement the medium, suggesting that the S-layer protein was indeed responsible for the protection observed (Fig. 2).

To obtain a visual assessment of the interaction between the S-layer and antimicrobial peptides, strains NA1000 and JS1013 were treated with biotinylated LL-37 (B-LL-37) for 30 min. Subsequently, the cells were washed with 0.25× PBS (2.5 mM sodium phosphate [pH 7.2], 37.5 mM NaCl) and incubated on ice for 30 min with streptavidin-AlexaFluor 488 conjugate (green label) (1:200 dilution) to label peptide B-LL-37. To visualize the bacterial S-layer, the cells were incubated for 30 min with polyclonal rabbit anti-RsaA antibody (1:200 dilution) and then washed by centrifugation and resuspension. Then a goat anti-rabbit antibody conjugated with AlexaFluor 568 (red label) was added to the cells (1:200 dilution) for 30 min. After three washing steps, the cells were pelleted and suspended in a

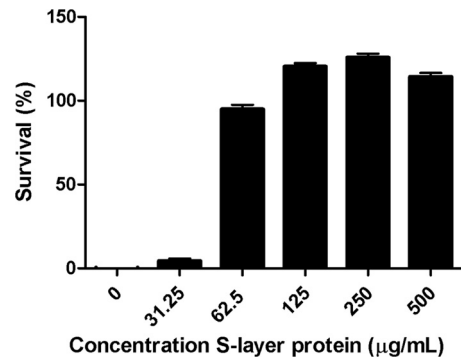


FIG 2 Exogenous addition of S-layer protein RsaA to JS1013 (S-layer negative) in culture restores protection against antimicrobial peptides. To obtain RsaA, an attachment-defective strain (JS4032) containing expression plasmid p4B:rsaA600 was grown in M15 minimal medium (11) at 30°C with shaking. At an optical density (600 nm) of 1.0, cells were pelleted and the supernatant collected and dialyzed (Spectra/Por dialysis membrane; molecular weight cutoff, 6,000 to 8,000) in water overnight. The presence and purity of protein were confirmed before and after dialysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Then the supernatant was lyophilized, and the lyophilized protein was used after suspension in water. The broth microdilution (24) protocol was used in which strain JS1013 was treated with increasing concentrations of RsaA and a single concentration of LL-37 (2 µg/ml, i.e., the MIC for JS1013). After 24 h, optical densities (600 nm) were measured. The data correspond to one of two independent experiments that showed similar restoration of protection upon addition of exogenous RsaA protein.

small amount of 20 mM phosphate buffer containing 50% glycerol and 2% *n*-propyl gallate. Peptide B-LL-37 and the S-layer were visualized using epifluorescence microscopy. S-layer-negative cells (JS1013) exhibited increasing green fluorescence at peptide levels between 0.5 µg/ml and 4 µg/ml, implying that B-LL-37 had been taken up by cells (Fig. 3). After treatment with 8 µg/ml of peptide, no JS1013 cells were observed by phase-contrast microscopy (data not shown). In contrast, in strain NA1000, B-LL-37 labeling was observed only at 8 µg/ml (Fig. 3). The lack of green labeling at lower concentrations likely indicated that the peptide might have interacted with the S-layer but was washed away during the washing steps.

In conclusion, we have identified a new role for the bacterial S-layer as a protective barrier against antimicrobial peptides. This protection could be due to the low isoelectric points of S-layer proteins [e.g., *C. crescentus* RsaA has a pI of 3.46 (7)], since except for the S-layers of *Lactobacilli*, all known S-layers have low isoelectric points. Thus, interactions between the positive charges of the peptide and negatively charged residues on the S-layer (carboxylate side chains of aspartates and glutamates) could prevent the peptides from reaching the outer membrane.

We propose that one of the reasons behind the selection of the S-layer throughout evolution is its importance as a resistance mechanism that allows bacterial survival when antimicrobial peptides are present in the environment. As antimicrobial peptides are produced by virtually every living organism, including bacteria themselves, we would expect that bacteria living in oligotrophic environments might encounter antimicrobial peptides. The S-layers present in pathogenic bacteria such as *B. anthracis*, *C. fetus*, *A. salmonicida*, and *B. sphaericus* may also serve as resistance barriers against antimicrobial peptides in environmental, clinical, and experimental settings.

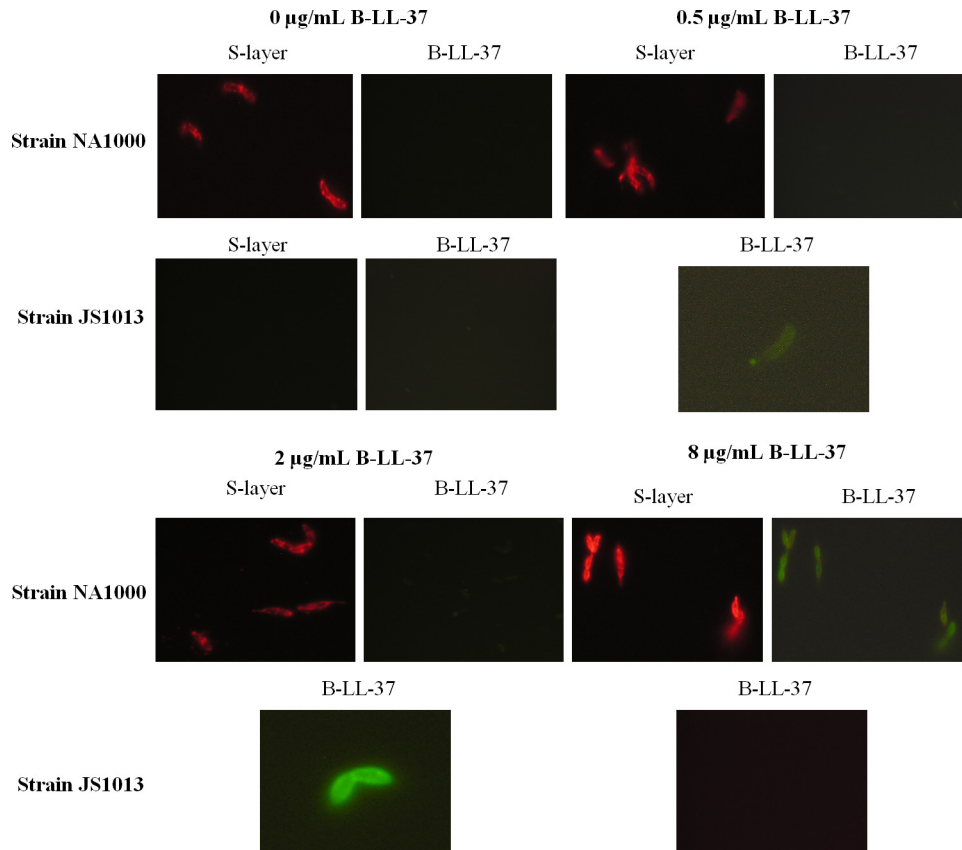


FIG 3 Exogenously added biotinylated peptide LL-37 was unable to enter the outer membrane of S-layer-positive cells (strain NA1000) at concentrations below 8 $\mu\text{g}/\text{ml}$. The S-layer was labeled with a polyclonal rabbit anti-RsaA antibody and secondary goat anti-rabbit IgG conjugated to AlexaFluor 568, and the exogenously added peptide B-LL-37 was labeled with streptavidin-AlexaFluor 488.

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