Functional Characterization of a Newly Identified Group B Streptococcus Pullulanase Eliciting Antibodies Able to Prevent Alpha-Glucans Degradation

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Abstract

Streptococcal pullulanases have been recently proposed as key components of the metabolic machinery involved in bacterial adaptation to host niches. By sequence analysis of the Group B Streptococcus (GBS) genome we found a novel putative surface exposed protein with pullulanase activity. We named such a protein SAP. The sap gene is highly conserved among GBS strains and homologous genes, such as PulA and SpuA, have been described in other pathogenic streptococci. The SAP protein contains two N-terminal carbohydrate-binding motifs, followed by a catalytic domain and a C-terminal LPXTG cell wall-anchoring domain. In vitro analysis revealed that the recombinant form of SAP is able to degrade α-glucan polysaccharides, such as pullulan, glycogen and starch. Moreover, NMR analysis showed that SAP acts as a type I pullulanase. Studies performed on whole bacteria indicated that the presence of α-glucan polysaccharides in culture medium up-regulated the expression of SAP on bacterial surface as confirmed by FACS analysis and confocal imaging. Deletion of the sap gene resulted in a reduced capacity of bacteria to grow in medium containing pullulan or glycogen, but not glucose or maltose, confirming the pivotal role of SAP in GBS metabolism of α-glucans. As reported for other streptococcal pullulanases, we found specific anti-SAP antibodies in human sera from healthy volunteers. Investigation of the functional role of anti-SAP antibodies revealed that incubation of GBS in the presence of sera from animals immunized with SAP reduced the capacity of the bacterium to degrade pullulan. Of interest, anti-SAP sera, although to a lower extent, also inhibited Group A Streptococcus pullulanase activity. These data open new perspectives on the possibility to use SAP as a potential vaccine component inducing functional cross-reacting antibodies interfering with streptococcal infections.

Introduction

The use of carbon sources is essential to the ability of bacteria to colonize the host and potentially cause disease in humans. In particular, highly polymerized α-glucan polysaccharides, such as starch and glycogen, are most likely to be found in environmental niches. Indeed, it is known that dietary-derived starches are very abundant in the human colon [1,2,3], while glycogen is deposited in large amount in the vaginal epithelium during times of high estrogen availability [4,5]. Recent reports using in vivo models of colonization showed a correlation between the expression of proteins involved in sugars metabolism and virulence. For example, the malt-o-oligosaccharide/maltodextrin–binding component of the Group A streptococcus malt-o-oligosaccharide ABC transporter has been shown to be directly involved in virulence in a mouse model of oropharynx infection [6]. More recently, Shelburne et al. demonstrated that in human saliva the transcript levels of several GAS carbohydrate utilization proteins other than glucose are highly expressed [7]. In addition, a signature-tagged mutagenesis study on S. pneumoniae (SPN) highlighted that a number of α-glucan–active enzymes seems to be virulence factors in a mouse model of lung infection [8].

Because of the complex structures of highly polymerized α-glucans, bacteria require an appropriate combination of enzymes for de-polymerization to oligo- and monosaccharides. Among these enzymes are ascribed pullulanases. Pullulanases have a glycosidic hydrolase activity towards α-glucan polysaccharides and are considered key extracellular components in bacterial metabolism. GAS and Streptococcus pneumoniae (SPN) pullulanases, named PulA and SpuA respectively, have been recently described [9,10]. They are anchored to the cell wall at their C termini by an LPXTG motif and possess a modular structure harboring a carbohydrate binding motif belonging to family 41 (CBM41) well classified into 47 families on the basis of amino acid sequence [12]. CBMs are currently classified into 47 families on the basis of amino acid sequence [12]. In particular, family 41 in the CBM classification was identified for the first time in a pullulanase enzyme of the marine bacterium Thermotoga maritima and it shares a high specificity for α-glucans. Of interest, PulA has been described to have multifunctional activities as the capability to hydrolyze pullulan, a linear polysaccharide of bacterial adaptation to host niches. By sequence analysis of the Group B Streptococcus (GBS) genome we found a novel putative surface exposed protein with pullulanase activity. We named such a protein SAP. The sap gene is highly conserved among GBS strains and homologous genes, such as PulA and SpuA, have been described in other pathogenic streptococci. The SAP protein contains two N-terminal carbohydrate-binding motifs, followed by a catalytic domain and a C-terminal LPXTG cell wall-anchoring domain. In vitro analysis revealed that the recombinant form of SAP is able to degrade α-glucan polysaccharides, such as pullulan, glycogen and starch. Moreover, NMR analysis showed that SAP acts as a type I pullulanase. Studies performed on whole bacteria indicated that the presence of α-glucan polysaccharides in culture medium up-regulated the expression of SAP on bacterial surface as confirmed by FACS analysis and confocal imaging. Deletion of the sap gene resulted in a reduced capacity of bacteria to grow in medium containing pullulan or glycogen, but not glucose or maltose, confirming the pivotal role of SAP in GBS metabolism of α-glucans. As reported for other streptococcal pullulanases, we found specific anti-SAP antibodies in human sera from healthy volunteers. Investigation of the functional role of anti-SAP antibodies revealed that incubation of GBS in the presence of sera from animals immunized with SAP reduced the capacity of the bacterium to degrade pullulan. Of interest, anti-SAP sera, although to a lower extent, also inhibited Group A Streptococcus pullulanase activity. These data open new perspectives on the possibility to use SAP as a potential vaccine component inducing functional cross-reacting antibodies interfering with streptococcal infections.
GBS Pullulanase Activity

The recombinant form of SAP shows a specific pullulanase enzymatic activity

The sap gene from the COH1 strain, without the signal sequence and the cell-wall anchoring region, was cloned into pET21b(+), expression vector. As shown in Fig. 2A, two main bands of 130 and 98 kDa were observed on SDS-PAGE gel, suggesting that two forms of the protein were being produced in E. coli. This is in agreement with previous data reported in the literature [21,22] and our data (Bombaci et al., unpublished observations) indicating the same protein pattern for recombinant PulA. On the basis of N-terminal sequencing analysis of the low MW form of SAP, which revealed the MKVQPNDYVF motif, we predicted a second putative GTG translational start codon within the COH1 sap ORF at position +1036 and a possible Shine-Dalgarno region (5'-AGGAGA-3') 4 bp upstream of this point. The resulting translation product obtained from this start site yield a smaller protein lacking both CBMs. A mixture of the high and low molecular weight forms of SAP (H+L) was purified by affinity chromatography. Moreover, by anionic exchange chromatography, we were also able to separate SAP (L) from SAP (H+L). Both SAP recombinant preparations were used to demonstrate that a pullulanase enzymatic activity was associated to the protein.

The capacity of recombinant SAP to catalyze the degradation of α-glucan polysaccharides was tested by 3,5-dinitrosalicylic (DNS) acid assay (see Experimental procedures). As shown in Fig. 3A, recombinant SAP was active on pullulan, glycogen type IX from bovine liver, amylopectin and starch, in which glucose residues are linked by both α-1-4 and α-1-6 glycosidic linkages. On the contrary, SAP was unable to catalyze amylase, which is a linear glucose polymer carrying exclusively α-1-4 glycosidic linkages. For the specific cleavage of α-1-6 glycosidic linkages, we hypothesize that SAP is likely to be a Type I pullulanase [23].

Of interest, comparison of SAP(H+L) versus SAP(L) preparations, showed that they were both active on pullulan, starch and amylopectin, while only SAP(H+L) was able to degrade glycogen. This finding suggests that the CBM contributes to the specific interaction with glycogen, in agreement with previous reports [8,10].

In addition, we compared the enzymatic activity of SAP versus GAS pullulanase (PulA) using different carbohydrates as substrates. As shown in Fig. 3B, no statistically differences were observed among SAP(H+L) and PulA for the capacity to degrade pullulan, glycogen, amylopectin and starch. These data postulate that, although the overall sequence conservation is around 60% of identity, pullulanase enzymatic activity well correlates between SAP and PulA.

SAP is a Type I pullulanase that generates maltotriose residues

In order to confirm the classification of SAP as a type I pullulanase we evaluated the modifications of the structure of pullulan after incubation with SAP by NMR spectroscopy. Fig. 4A shows the proton NMR spectra of pullulan incubated in the presence or absence of SAP(H+L). All the signals have been assigned by using 1H-1H 2D NMR scalar chemical shift correlation spectroscopy, which gave results in agreement with the assignments reported in the literature [24]. The NMR chemical shift of selected signals, particularly looking at the anomeric region (about from 5.8 to 4.5 ppm), has been used to monitor the structural degradation of the polysaccharides. The 1H NMR spectrum of pullulan after the addition of SAP(H+L) (Fig. 4A) contains the C1 protons present in the starting material and a new anomeric α-linked signal [H$_{\alpha1}$ (C Maltotriose)] at 5.33 ppm,
Figure 1. Modular organization of the SAP protein from the COH1 strain. (A) In green the signal peptide sequence. In gray the two tandems CBM41, in yellow the catalytic domain (glycoside hydrolase family 13) and in red the C-terminal LPXTG cell-wall anchoring motif. (B) Sequence alignments of individual CBM41s from GBS COH1 (SAN_1346), 515 (SAL_1339), CJB111 (SAM_1238), A909 (SAK_1302), NEM (gbs_1288), H36B (SAI_1308), 2603 V/R (SAG_1216) strains, GAS SF370 strain (Spy_1972) and SPN TIGR4 strain (SP_0268). The conserved residues present in the α-
generated by enzymatic cleavage of the glycosidic bonds. The reducing end β-linked signal [H₁,β(C-Maltotriose)] is not detectable due to the overlapping with the major HDO peak. Since the peak integral ratio between [H₁,β(C-Maltotriose)+H₁β(C-Maltotriose)] and H₁α [α(R β Maltotriose)] is 1:2, we can conclude that the SAP cleaves α-(1,6) glycosidic linkages between the units A and C generating maltotriose units [25,26].

Glycogen molecular size distribution before and after the addition of SAP(H+L) was instead determined by size exclusion chromatography. As reported in Fig. 4B, the intensity of glycogen RI signal decreased after 20 min from the addition of SAP(H+L). From these data we can conclude that SAP is also active on glycogen as confirmed by DNS acid assay.

Alpha-glucans modulates SAP expression on bacterial surface

We observed that when GBS was grown in THB medium, a rich medium normally used to culture GBS in laboratory, SAP was not expressed on bacterial surface (data not shown). Since bacterial pullulanases are known to be regulated by specific carbon sources [27], we hypothesize that the amount of glucose in THB medium (2 g/L) may down-regulate SAP expression. Therefore, expression analysis was performed by using a Complex Medium (CM) to which different α-glucans were added. We investigated the mechanisms of regulation of SAP expression by RT-PCR, Immuno-Electron Microscopy (IEM), confocal microscopy, FACS and Western blotting (WB). As expected, SAP messenger RNA transcript was undetectable when GBS was grown in CM supplemented with different carbohydrates. As expected no growth differences were observed among these strains when glucose or maltose, that are not pullulanase substrates, were added to the CM (Fig. 6A and B). On the other hand, the presence of pullulan or glycogen in CM while did not affect the capacity of the sap mutant strain to replicate, increased the growth rate of the wild type strain (Fig. 6C and D).

In order to confirm that the capacity of GBS to hydrolyze α-glucans is associated to a an increased expression of SAP, we compared a total protein extract derived from COH1 wild type strain versus COH1Δsap strain for the ability to grow in CM supplemented with different carbohydrates. As expected no growth differences were observed among these strains when glucose or maltose, that are not pullulanase substrates, were added to the CM (Fig. 6A and B). On the other hand, the presence of pullulan or glycogen in CM while did not affect the capacity of the sap mutant strain to replicate, increased the growth rate of the wild type strain (Fig. 6C and D).

A SAP deficient mutant strain shows an impaired capacity to grow in pullulan and glycogen containing complex medium

To investigate whether SAP enzymatic activity is essential for bacterial replication in the presence of α-glucans, we compared COH1 wild type strain versus COH1Δsap strain for the ability to grow in CM supplemented with different carbohydrates. As expected no growth differences were observed among these strains when glucose or maltose, that are not pullulanase substrates, were added to the CM (Fig. 6A and B). On the other hand, the presence of pullulan or glycogen in CM while did not affect the capacity of the sap mutant strain to replicate, increased the growth rate of the wild type strain (Fig. 6C and D).

In order to confirm that the capacity of GBS to hydrolyze α-glucans is associated to an an increased expression of SAP, we compared a total protein extract derived from COH1 wild type and COH1Δsap strains grown in the presence of different carbohydrates, for the ability to degrade pullulan. As shown in the Fig. 7, pullulanase activity was only observed in the protein extracts of COH1 wild type grown in the presence of sugars inducing SAP
expression, such as pullulan and glycogen. These findings suggest that GBS utilize α-glucans as a carbon energy source and that pullulanase is indispensable to this activity.

SAP is recognized by human sera

Recent reports revealed that both sera from patients with GAS and SPN infections contained antibodies reactive with pullulanases [28,29]. In order to assess whether human sera recognized recombinant SAP, we tested 4 sera from normal healthy volunteers. By quantitative ELISA we found that all sera tested showed antibody titers against SAP(H+L) and that IgG concentrations were in a range of 54.8–116.7 μg/ml, with a geometric mean concentration of 76.7±31.9 μg/ml. Of interest, antibody titers against SAP(L) were lower compared to SAP(H+L) (Fig. 8A). The specificity of the assay was confirmed by competitive ELISA using the purified recombinant SAP protein as an inhibiting antigen (Fig. 8B). The addition of an unrelated recombinant GBS surface protein did not inhibit antibody binding to the SAP protein in this assay (Fig. 8B). These findings other than indicating the specificity of the antibody response towards SAP, suggest that the CBMs might be important for the immunogenicity of the protein.

Anti-SAP antibodies block SAP and PulA enzymatic activity

In order to test whether the immunoglobulin-mediated response towards SAP impaired bacterial metabolic activity versus α-glucans, we tested by DNS acid assay the capacity of mouse and rabbit anti-SAP sera to prevent GBS pullulanase activity. We performed dose-dependent experiments incubating SAP-expressing bacteria with different sera dilutions in a range between 0.1–2%. As shown in Fig. 9, we observed that a SAP mouse antiserum was able to block in a dose dependent fashion the ability of GBS COH1 strain to degrade pullulan up to 80% of the initial activity (Fig. 9A). As expected, the addition in the assay of two unrelated sera did not inhibit GBS SAP activity (Fig. 9A). Similar results were obtained performing the experiments using glycogen as a substrate (data not shown). Depletion of specific anti-SAP antibodies by absorbing the anti-SAP serum to a CNBR resin coated with recombinant SAP, resulted in no inhibition of GBS capacity to catabolize pullulan (Fig. 9A). As a control, the absorbed anti-SAP serum lost the capacity to recognize the recombinant form of SAP in immunoblotting assay (data not shown). These data clearly postulate that anti-SAP antibodies mediate in vivo prevention of GBS pullulanase activity. The attempt to reduce SAP enzymatic activity by adding to bacteria human sera containing anti-SAP antibodies (up to a concentration of 10%) or anti-SAP antibodies purified from human sera was unsuccessful (data not shown). We hypothesize that the quantity and quality of anti-SAP antibodies derived from adult healthy volunteers that have been in contact with GBS, is not sufficient for our in vitro assay. Unfortunately, no sera from convalescent patients or with GBS invasive disease are at the moment available in our laboratory.

Since SAP CBM appears to be a very immunogenic domain (Fig. 8A) and that it is highly homologous among streptococcal pullulanases, we decided to test whether anti-SAP specific sera were able to reduce the capacity of GAS to catabolize pullulan. For these experiments we used GAS SF370 strain, which in the presence of pullulan expresses PulA, as demonstrated by both Western Blotting and FACS analysis (data not shown). As shown in Fig. 9B, we observed that a SAP antiserum was able to reduce the activity of GAS to catabolize pullulan, up to 50% of the initial activity. These data confirm our hypothesis that the activity of anti-pullulanase antibodies may be cross-species.

Discussion

The increasing need of new vaccine-based preventive strategies replacing the antibiotic prophylaxis used for eradicating GBS colonization of the genital tract of pregnant women, has recently led to identification of antigens conferring a broad protection in mice [30]. The discovery of novel immunogenic virulence factors has also opened new perspectives to tackle GBS-associated infections [31,32]. In this context, we considered of importance the understanding of GBS genes involved in adaptive metabolism of the bacterium. Indeed, the mechanisms underlying the capacity of GBS to use complex carbon sources available at site of colonization are largely undefined. Several lines of evidence are now indicating that degradation of complex host-derived carbo-
Figure 4. Analysis of SAP(H+L) enzymatic activity on pullulan and glycogen. (A) NMR spectra indicate the generation of maltotriose units after the addition of SAP(H+L) to the reaction mixture containing pullulan. Pullulan NMR spectra were recorded on the native polysaccharide (-SAP) and after the addition of the recombinant enzyme (+SAP). NMR experiments were recorded at 25°C on Bruker Avance 600 MHz spectrometer and using 5-mm probe (Bruker). For details see the Experimental Procedures section. (B) SEC-HPLC analysis indicates that SAP(H+L) is active on glycogen. Two chromatograms were recorded at 214nm, one on the native glycogen polysaccharide (black line) and the other 1 h later the addition of SAP(H+L) (blue line). A gel filtration analytical column with a fractionation range of Mw PEG/PEO 2×10^5–3×10^5 Da was used. For details see the Experimental Procedures section.

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hydrates is crucial to bacterial virulence. In particular, analysis of the transcriptome of GAS in a mouse soft tissue infection model developed by J. Musser’s group [15], identified a series of genes highly expressed during adaptive metabolic responses triggered by nutrient signals and hypoxic/acidic conditions in the host. Of interest, among them were identified genes related to amino acid and maltodextrin utilization such as PulA. Indeed, GAS metabolism of complex host-derived carbohydrates may be particularly important during soft tissue infections because of abundant host glycoproteins and host cell contents released during cell lysis. GAS allows transcription of carbohydrate utilization genes and virulence factors also in other low-glucose environmental conditions such as those found in human oropharynx and saliva [15]. In agreement with our findings, it has been recently demonstrated that GAS pullulanase is up regulated in bacteria grown in human saliva, where glucose levels are low, compared to the growth in nutrient rich medium [7]. On this basis, we hypothesize that similar expression patterns may be induced by GBS during colonization of lower gastro-intestinal and female genital tracts, known to be poor in glucose but rich in α-glucans [2,4]. Our data indicate that SAP-expressing GBS strains actively degrade glycogen and that the recombinant form of SAP lacking both CBMs loses activity versus this substrate. Based on these evidence, we propose that SAP may have a role in vivo during the establishment of vaginal colonization and confirms the specificity of CBMs for glycogen [10]. Moreover, in silico analysis of GBS available genomes revealed that SAP is the only surface associated protein containing glycosidic domains (Santi et al., unpublished results) and since SAP appears to be the only enzyme expressed by GBS capable to catabolize α-glucans, we suggest that this enzyme may be vital for GBS permanence in environmental niches poor in glucose. We are currently testing this hypothesis, and preliminary data have shown that deletion of the sap gene reduces the capacity of GBS to colonize the vagina in a mouse model of infection (Pezzicoli et al., unpublished results).

Table 1. Exposure of SAP on bacterial surface in the presence of different carbohydrates.

| Carbohydrates | COH1 | COH1Δsap | COH1-13 |
|---------------|------|----------|---------|
| Glucose       | 7**  | 0        | 62      |
| Maltose       | 56   | 0.000    | 308     |
| Pullulan      | 232  | 0        | 453     |
| Glycogen      | 255  | 0        | 452     |

*Numbers indicate the delta mean of fluorescence relative to bacteria incubated with a SAP immune serum versus bacteria incubated with a pre-immune serum.

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Emerging theories on human-microbe mutualism suggest that the mechanisms that underlie microbial community structure and host–symbiont relationships should be considered for planning prevention strategies for human health. Indeed, as recently proposed by David A. Relman [33], it should be investigated the role of microbial communities, and not just individual species,
Figure 6. The capacity of GBS to grow in pullulan and glycogen depends on SAP expression. The graphs represent the growth curves relative to GBS COH1 wild type strain and COH1Δsap mutant strain grown in complex medium alone or with the addition of glucose (A), maltose (B), pullulan (C) and glycogen (D). White circles indicate the COH1 wild type strain incubated in the presence of sugars, while white squares the same strain incubated in complex medium alone. Black circles represent the COH1Δsap strain grown in complex medium supplemented with sugars, while black squares are relative to the same strain grown in complex medium alone. A typical experiment, out of 4 performed giving identical results, is shown. (E–F) Comparison of CFU/ml recovered after growing GBS COH1 wild type and COH1Δsap for 3 h in the presence of pullulan (E) or glycogen (F). The data are the mean of 3 independent experiments ± SD. The asterisk indicates a significant difference between values (p<0.01).

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Figure 7. Detection of pullulanase activity in GBS total extracts.
Bacteria grown in a complex medium supplemented with the indicated sugars were used to prepare total extracts. Bacterial extracts were then incubated with pullulan and pullulanase enzymatic activity measured by DNS acid assay. The asterisks indicate a significant difference between the activity of the wild type strain versus the mutant strain derived extracts (p<0.01). The data are the mean of 3 independent experiments ± SD.

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as pathogens. In this perspective, reduction of the fitness of pathogens by affecting their metabolic activity towards essential nutrients may be more effective than a general bactericidal activity, as the one offered by an antibiotic treatment. Vaccines able to specifically prevent infection from multiple microorganisms are highly desirable. In this context, we found that anti-SAP sera other than preventing GBS catabolism of pullulan, significantly reduce pullulanase activity in a GAS strain expressing PulA is of extreme importance. In particular, we hypothesize that the immunization of individuals with SAP will raise antibodies, which by impairing the metabolic activity of pathogenic streptococci might shift the equilibrium that regulates the colonized human niches in favor of the commensal population.

In conclusion, the evidence reported in this paper may draw up the basis for preventing streptococcal infections by using immunogenic metabolic enzymes as target molecules for vaccine development. The fact that, at least for pathogenic streptococci, such enzymes are well conserved opens new perspectives in the development of strategies preventing infections from multiple species.

Materials and Methods

Sequence analysis

The alignment of SAP protein encoded by sap gene from 2603 V/R (TIGR Accession SAG_1216), 515 Lai (SAL_1339), NEM316 (gbs1288, H36B (SAI_1308), CJB111 (SAM_1238), A909 (SAK_1302) and COH1 (SAN_1346) strain as well as SpuA (SP_0268) and PulA (Spy_1972) was performed using ClustalW [34].

Bacterial strains and growth conditions

S. agalactiae strains COH1 serotype III was used in this study. Escherichia coli DH5α and DH10BT1 were used for cloning purposes and E. coli BL21 (DE3) for expression of SAP fusion protein. S. agalactiae was cultivated at 37°C in Todd-Hewitt broth (THB) up to desired OD600. E. coli was grown in Luria-Bertani broth (LB); E. coli clones carrying the plasmids pJRS233 or pET21(b)+ and derivatives were grown in the presence erythromycin (400 μg/mL) or ampicillin (100 μg/mL), respectively. The complex medium (CM), 10 g/l proteose peptone, 5 g/l trypticase peptone, 5 g/l yeast extract, 2.5 g/l KCl, 1mM Urea, 1mM Arginine, pH 7.0) was used for GBS growth with defined carbon sources. The sugar concentrations were 1% final. To evaluate growth in CM, GBS was initially grown to log phase (OD600 0.5) in THB. The cells were harvested by centrifugation, washed twice in an equivalent volume of phosphate-buffered saline (PBS) and diluted 1 to 50 in CM. Growth was monitored spectrophotometrically at a wavelength of 600 nm.

SAP recombinant protein expression and purification

In order to express the recombinant form of SAP, the open reading frame of the sap gene from S. agalactiae COH1 serotype III was used as a template. The construct was amplified by PCR using specific primers GBS5F and GBS6R introducing Ndel and Xhol restriction enzyme sites (Table 2).

The PCR products were cloned into the pET21(b)+ vector and the plasmid transformed in E. coli BL21 (DE3) cells. BL21 (DE3) cells were grown in LB-Amp (100 μg/mL ampicillin) and induced with IPTG at a final concentration of 1 mM for 3 hours. The resulting biomass was suspended in 0.3 M NaCl, 50 mM Na-PO₄ buffer, pH 8.0 and cells were lysed by enzymatic digestion. The sample was then loaded onto a His-Trap Ni-Activated Chelating Sepharose FF column (Amersham Biosciences, Milan, Italy) at a flow rate of 5 ml/min. Bound proteins were then eluted from the column by running a gradient from 0 to 50% of 500 mM Imidazole, 0.3 M NaCl, 50 mM Na phosphate buffer, pH 8.0 in 12 CV. The IMAC eluted material was collected in 2.5-ml fractions and those ones containing the SAP-His protein pooled. An anionic exchange chromatography was used to separate the two forms of SAP. The pooled fractions from Ni-IMAC were dialyzed against 30mM TRIS, pH 8.0 and then loaded on to a HiTrap Q HP 5 ml column (GE) to further purify the two forms of recombinant SAP. The purification was achieved by running a gradient from 0 to 50% 1M NaCl in 30mM TRIS, pH 8.0 in 16 CV at 5 ml/min. The collected fractions were analyzed by SDS-PAGE (Criterion™ pre-cast gel, 200V, 55 min) and pooled according to apparent MW. The final preparation of the protein was obtained in PBS, pH 7.4 after dialysis.

Construction of COH1 sap deletion mutant

The sap gene was deleted in GBS strain COH1, according to the procedure previously described [35]. The in-frame deletion fragment was obtained by Splicing Overlap Extension (SOE) PCR using the primers P1, P2, P3 and P4 (Table 2). The XhoI restriction enzyme cleavage sites were incorporated at the 5′-end of the primer to clone the fragment into the XhoI-digested pJRS233 plasmid. After cloning the in frame deletion fragment in pJRS233, the plasmid pJR9233Δsap was obtained.

The plasmid pJR9233Δsap was then transformed into the COH1 strain by electroporation and transformants were selected after growth at 30°C on agar plates containing 1 mg/ml erythromycin. Transformants were then grown at 37°C with erythromycin selection as previously described [36]. Integrant strains were serially passaged for 3 days in liquid medium at 30°C without erythromycin selection to facilitate the excision of plasmid pJR9233Δsap, resulting in the sap deletion on the chromosome. Dilutions of the serially passaged cultures were plated onto agar plates, and single colonies were tested for erythromycin sensitivity to confirm the excision of pJR9233Δsap. The resulting strain was named COH1Δsap.

Bacterial extracts

GBS protein extracts were prepared by growing bacteria up to OD600 0.4 in CM plus sugars, washed in PBS and incubated for
1 h at 37°C in 500 µl of Tris-HCl 50 mM (pH6.8) containing protease inhibitors and 400 U/ml of mutanolysin (SIGMA, MO, USA). The bacterial suspension was then pelleted and the supernatants containing peptidoglycan associated proteins used for western blotting analysis of SAP. In order to prepare GBS extracts relative to the secreted protein fraction, supernatant of bacteria cultures grown to OD600 0.4 were collected. Proteins in 1 ml of supernatant were precipitated with 10% of trichloroaceticacid (TCA) for 1 hr at 4°C. Protein were then pelletted, washed with cold acetone and resuspended in Tris-HCl pH 6.8.

RT-PCR

COH1 was grown in CM medium plus sugars up to OD600 0.4. Total GBS RNA was isolated using the Rnasy mini kit (Qiagen) according to manufacturer’s instructions, except that bacteria were lysed with 100 µl of lysozyme (30 mg/ml) in Tris-EDTA buffer and 2,000 U of mutanolysin, and the mixture was incubated for 15 min at 37°C. Quantification of the transcripts was completed by reverse transcription and semi-quantitative RT-PCR using ImPromII RT (Promega) following manufacturer’s instructions. Briefly 2 µg of sample and 0.5 µg of random hexamers were added to a final volume of 5 µl. Samples were incubated in a thermocycler (Biometra) at 70°C for 5 min followed by a quick chill at 4°C. The mixture was used in a 20-µl (total volume) cDNA synthesis reaction mixture comprising 4 µl of Improm-II 5× reaction buffer (Promega), 2.4 µl MgCl2 at 25 mM, 2 µl of dNTP mix (each dNTP at 2.5 mM), 0.25 µl of Rnasin RNase inhibitor (Promega) and 1 µl of Improm-II reverse transcriptase. The reaction was performed at 42°C for 60 min. In the negative controls, the reverse transcriptase was substituted with water. 2 µl of cDNA were then added to the PCR reaction consisting of 1× reaction buffer, 200 µM dNTP’s, 0.2 µM primer pair, 1 U PlatinumTaq dna polymerase (Invitrogen). GBS Gyrase A (GyrA) was used as an internal housekeeping control. PCR reactions consisted of a 7-min denaturation step 94°C, followed by a variable number of cycles. PCR products were electrophoresed through 2% agarose gels and images were acquired by laser densitometry (Gel-Doc Imaging System).
NMR analysis

Samples of pullulan (Sigma) and glycogen (Sigma) were prepared by dissolving polysaccharide powder (10 mg) in 0.7 mL of deuterated PBS buffer at pH 7.2 (D2O, 99.9% atom D – Aldrich was used) to a uniform concentration. Samples were therefore transferred to 5-mm NMR tubes (Wilmad Glass. Co.). 70 μL of SAP were therefore added to the pullulan and glycogen samples in the NMR tubes. For every sample, two NMR spectra were recorded, the first on the native polysaccharide and the second 1 hour later (incubation at 25°C) after the addition of enzyme. Samples of maltose (Sigma) and maltotriose were also prepared by dissolving 10 mg of powder in 0.7 mL of deuterated PBS buffer at pH 7.2. 1H NMR experiments were recorded at 25°C on Bruker Avance 600 MHz spectrometer and using 5-mm probe (Bruker). For data acquisition and processing XWINNMR software package (Bruker) was used. 1-D proton NMR spectra were recorded using a standard one-pulse experiment. 64 scans were collected and averaged, giving a total acquisition time of ca. 10 min. The transmitter was set at the HDO frequency, collecting 32 k data points over a spectral window of 6,000 Hz. 1H NMR spectra were obtained in quantitative matter using a total recycle time to ensure a full recovery of each signal (5x Longitudinal Relaxation Time T1). Spectra were Fourier Transformed to 32 k data points after applying a 0.2 Hz line broadening function and referenced relative to the HDO resonance at 4.79 ppm.

Size Exclusion Chromatography (SEC)-HPLC analysis

Samples of glycogen were prepared by dissolving polysaccharide powder (1 mg) in 0.1 mL of PBS at pH 7.2 to a uniform concentration. Samples were therefore transferred to 1 mL vials (Waters). 10 μL of SAP were therefore added to the glycogen sample. Two chromatograms were recorded, the first on the native polysaccharide and the second 1 hour later (incubation at 25°C) after the addition of enzyme. A TSK G4000PW (TosoHaas) gel filtration analytical column (7.5 mm x 30.0 cm) with a fractionation range of Mw PEG/PEO 2,000–3 x 105 Da was used. Samples were loaded onto the gel filtration column and eluted isocratically in 100 mM sodium phosphate + 100 mM NaCl buffer pH 7.2 at a flow rate of 0.5 ml min^-1 for 50 min. The elution was monitored with a Ultimate 3000 Photodiode Array detector (Dionex) coupled with the Ultimate 3000 HPLC system (Dionex). For data acquisition and processing Chromeleon software package (Dionex) was used.

Fluorescence-activated cell sorter analysis

In order to quantify the exposure of SAP on the bacterial surface, GBS was grown up to OD600 0.4 in CM with 1% sugar, fixed with 1% PFA for 20 min at RT and incubated with mouse anti-SAP serum or mouse anti-PBS serum (negative control) in 0.1% BSA plus 20% of Normal Calf Serum (NCS) for 1 h at 4°C. Bacteria were then washed in PBS containing 0.1% BSA and incubated with the phcoerytrin (PE) conjugated secondary antibodies (Jackson Immuno Research Inc., PA, USA) for 45 min at 4°C. After washing bacteria were resuspended in

Table 2. List of primers used in the study.

| Primer | Sequence (5’ to 3’) |
|--------|---------------------|
| GBS5F  | CTAGCTACGCGAAGAAGTAAGTGTTTCTC |
| GBS5R  | CCGGTCGAGATTAGCTTATTTGTCAGA |
| COH1\ Δsap | |
| P1     | CCGGTCGAGTCATCTACACACGGCATTTCCTCC |
| P2     | TCCAGTTTTGCGAAGGGAGTAGTTTTATGG |
| P3     | TTGCAAAATACCTCTCTGCCAAAACCTGGAGATAA |
| P4     | CCGGTCGAGTCTCTAATGCTGTCTAACC |

F corresponds to forward primer and R to reverse primer. Restriction sites are underlined.

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200 μl of PBS and analyzed by a FACScan flow cytometer (Becton Dickinson) by using FlowJo software program.

Immunogold labeling and electron microscopy

GBS strains COH1 were grown at 37°C up to OD600 0.3 (exponential phase) in CM plus 1% sugars. Bacteria were then centrifuged for 10 min at 3000 rpm (RT), washed and resuspended in 1 ml of PBS. Formvar-carbon-coated nickel grids were floated on drops of GBS suspensions for 5 min. The grids were then fixed in 2% PFA for 5 min, and placed in blocking solution (PBS containing 1% normal rabbit serum and 1% BSA) for 30 min. The grids were then floated on drops of primary antiserum against the SAP protein diluted 1:20 in blocking solution for 30 min at RT, washed with six drops of blocking solution, and floated on secondary antibody conjugated to 10-nm gold particles diluted 1:20 in 1% BSA for 30 min. The grids were examined using a TEM GEOL 1200EX II transmission electron microscope.

Confocal immunofluorescence microscopy

In order to visualize SAP on bacterial surface, COH1 was grown in CM plus sugars up to OD600 0.4 and washed in PBS. Bacterial pellet were fixed in 2% PFA for 20 min at RT and spotted on POLYSINE™ slides (Menzel-Glaser). The slides were then fixed in 2% PFA for 5 min, and placed in blocking solution (PBS containing 1% normal rabbit serum and 1% BSA) for 30 min. The grids were then floated on drops of primary antiserum against the SAP protein diluted 1:20 in blocking solution for 30 min at RT, washed with six drops of blocking solution, and floated on secondary antibody conjugated to 10-nm gold particles diluted 1:20 in 1% BSA for 30 min. The grids were examined using a Bio-Rad confocal scanning microscope.

3,5-dinitrosalicylic acid (DNS) assay

Pullulan activity was determined by measuring the enzymatic release of reducing groups from α-glucans by the DNS colorimetric method [37]. The mixtures contained 1% (w/v) pullulan, glycosan type IX, amylose, amylopectin or soluble starch (Sigma) dissolved in PBS (pH 7.0), and appropriately diluted enzyme in a total volume of 500 μL. After incubation at 37°C for 1 h, the reaction was stopped by addition of 1 mL of cold DNS buffer, followed by boiling for 15 min. 330 μL of a 40% potassium sodium tartrate (Rochele salt) solution was added to each tubes to stabilize the color. The release of reducing groups from α-glucans was determined by reading the absorption at 575 nm of the sample. The same sample without the enzyme was used to correct for non-enzymatic release of reducing sugars.

Serum-mediated inhibition of GBS and GAS pullulanase activity

GBS and GAS were grown at 37°C up to mid-late exponential phase (OD600 0.6) in THB and THY, respectively. Bacteria were then re-inoculated in CM containing 1% pullulan and grown to log phase (OD600 0.4) to allow the expression of pullulanases on bacterial surface. The cells were then harvested by centrifugation, washed twice with PBS and resuspended in PBS. Bacteria (5×1010 CFU) were pre-incubated with sera dilutions at 37°C for 15 min, then pullulan was added (1% final) and the incubation prolonged for other 2 hours. Samples were centrifuged and supernatants were used for the determination of reducing sugars by DNS acid assay.

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Author Contributions

Conceived and designed the experiments: IS AP MS. Performed the experiments: IS AP MB FB MM. Analyzed the data: IS AP MB FB MM JLT GG MS. Wrote the paper: IS AP FB GG MS.

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