Exogenous Sialic Acid Transport Contributes to Group B Streptococcus Infection of Mucosal Surfaces

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By sequence analysis of available group B streptococcus (GBS) genomes, we discovered a conserved putative operon involved in the catabolism of sialic acid, containing a tripartite transporter formed by two integral membrane components and a sugar-binding unit, named SAL0039. Expression analysis in the presence of different substrates revealed that SAL0039 was specifically upregulated by the presence of sialic acid and downregulated when bacteria were grown in human blood or in the presence of a high concentration of glucose. The role of SAL0039 in sugar transport was supported by the inability of the sal0039 deletion mutant strain to import exogenous sialic acid and to grow in semidefined medium supplemented with this sugar. Furthermore, in vivo evidence showed that the presence of exogenous sialic acid significantly increased the capacity of GBS to infect mice at the mucosal level. These findings suggest that transport of sialic acid may also contribute to GBS infections.

To successfully colonize human niches, pathogenic and commensal bacteria require the use of substances found at the site of colonization as carbon, nitrogen, and energy sources. Depending on the anatomical area, the nature and the availability of these compounds can vary enormously. Nonulosonic (sialic) acid is a 9-carbon sugar widely diffused in mammals; it is found as the terminal unit of polysaccharide chains associated with cellular surface–exposed glycoproteins and glycolipids and with secreted mucus proteins [1]. The most abundant and best characterized form of this sugar is N-acetylneuraminic acid (Neu5Ac), although >40 variants of the parent compound are present in nature. The presence of sialic acid on the surface of mammalian cells is important for cell–cell interaction and molecule–cell modulation processes [2], whereas in prokaryotes sialylation of surface polysaccharides confers protection against the host innate immune system [3]. A number of human pathogens are capable of endogenous sialic acid biosynthesis, whereas others, such as Haemophilus influenzae, acquire the sugar from the environment [4]. Indeed, several human pathogens (bacteria, fungi, and viruses) secrete sialidase enzymes that scavenge sialic acid residues from host glycoproteins. As a consequence of this, free sialic acid becomes available for bacterial species that can then use it for growth purposes, to sialylate their own capsule, or during biofilm formation [4–6]. Once monomeric sialic acid is released in the environment, bacteria must then capture this resource using specific transporters. High-affinity sialic acid transporters have been identified in different bacterial species. They can be divided into different functional classes: members belonging to the major facilitator superfamily; tripartite adenosine triphosphate (ATP)−independent periplasmic transporters; members of the sodium solute symporter family; ATP-binding cassette (ABC) transporters; and members of the sodium/proline family [7].

Group B streptococcus (GBS) is an extracellular mucosal pathogen behaving as a commensal organism that colonizes the lower gastrointestinal and genital tracts of about 20%–30% of healthy women. The capsular structures of every identified GBS serotype (Ia, Ib, II–IX)
share in common a terminal-linked sialic acid unit that has been associated, at least in part, with bacterial virulence because it interferes with phagocytic killing [8]. Although substantial evidence suggests that GBS may synthesize de novo sialic acid to produce a fully sialylated capsule [9, 10], the contribution of environmental sources as previously described [11]. Brie

**MATERIALS AND METHODS**

**Bacterial Strains and Growth Conditions**

*Streptococcus agalactiae* strain 515 serotype Ia was used in this study. Bacteria were cultivated routinely in Todd–Hewitt broth (THB) at 37°C with 5% carbon dioxide. A semidefinite complex medium (CM) was utilized for GBS growth with de

**Construction of sal0039 Deletion Mutant and Complemented Strain**

The sal0039 gene was deleted in GBS strain 515 as previously described [13]. The in-frame deletion fragment was obtained by splicing overlap extension polymerase chain reaction (PCR) using the primers P1, P2, P3, and P4 (Table 1). The XhoI restriction enzyme cleavage sites were incorporated at the 5’ end of P1 and P4 primers to clone the deletion fragment into the XhoI-digested pJRS233 plasmid.

The plasmid pJRS233Δsal0039 was then transformed into the 515 strain by electroporation, and transformants were selected by growing them at 30°C on agar plates containing 1 μg/mL erythromycin. Transformants were then grown at 37°C with erythromycin selection as previously described [14]. Integran strains were serially passaged for 4 days in liquid medium at 30°C without erythromycin selection to facilitate the excision of plasmid pJRS233, resulting in the sal0039 deletion on the chromosome. Dilutions of the serially passaged cultures were plated onto agar plates, and single colonies were screened by PCR to confirm the deletion of the gene. In-frame deletion was finally confirmed by genomic sequencing. For the complemented strain, the sal0039 gene was amplified from the 515 wild-type (515wt) genome and cloned into the shuttle vector pAM401/gbs80P + T as previously described [15].

**Animal Treatments and Evaluation of Bacterial Load**

Animal experiments were done in compliance with the current Italian law, and the treatments were approved by the Novartis Vaccines and Diagnostics Animal Ethics Committee. For the intranasal infection model, on day 0, groups of 5 female, 4-week-old, specific-pathogen-free BALB/c mice received into the nostrils 10 µL of the bacterial suspension containing 1.3 × 10^7 colony-forming units (CFUs) of either 515wt (2 groups) strain

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**Table 1**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
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<tr>
<td>515Δsal0039</td>
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</tr>
<tr>
<td>P1</td>
<td>CCCGCTCGAGACTGCAACACCATTAGACCA</td>
</tr>
<tr>
<td>P2</td>
<td>TTCAAGCATCTGGCCACGTAATTGACCTTTATCTCT</td>
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<td>P3</td>
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<td>C1</td>
<td>GTCATCGCGGCGGCTAAAAAGGGAATAGTTATG</td>
</tr>
<tr>
<td>C2</td>
<td>CTCTCTCTGATCTTTATTTAGCTGCTTTTTAATGCTATA</td>
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Primers used in this study. Restriction sites are underlined.
or 515Δsal0039 (2 groups) strain. The infection treatment was repeated on day 1. On day 2, 1 group infected with 515wt strain and 1 group infected with 515Δsal0039 strain received intranasal administration of 1 mg Neu5Ac in 10 µL of PBS, whereas the other 2 groups received PBS. On day 3, mice were euthanized and subjected to nasal and bronchoalveolar (lung) washes. Nasal wash was performed by flushing 0.5 mL saline through the nostrils, whereas lung wash was obtained in a final volume of 1 mL by 2 cycles of injection and extraction of 0.5 mL saline into the lungs. Washes were then serially diluted and plated for CFU counts. The limit of detection was 7 CFUs/mL. For the intravaginal infection model, groups of 10 female, 6-week-old, specific-pathogen-free BALB/c mice were synchronized by subcutaneous inoculation of 0.1 mg of β-estradiol valerate 48 hours before infection. On day 0, each animal received intravaginally 15 µL of bacterial suspension containing 1.7 × 10⁷ CFUs of either 515wt strain (2 groups), 515Δsal0039 strain (1 group), or the complemented 515Δsal0039 strain (1 group). On day 1, 1 group infected with 515wt strain and the groups infected with 515Δsal0039 and 515Δsal0039 strains received intravaginally 1 mg of Neu5Ac in 10 µL of PBS. The remaining group infected with 515wt strain received PBS only. On day 2, mice were euthanized, and vaginal washes were performed by flushing 0.5 mL of saline. Washes were then plated as above for CFU counts.

Statistical Analysis
Data of bacterial counts from nasal, lung, and vaginal washes were analyzed by 2-tailed Mann–Whitney U test. Values of P ≤ .05 were considered significant.

RESULTS
Group B Streptococcus Genome Contains a Putative Operon for Sialic Acid Metabolism
Pathogenic and commensal bacteria can utilize exogenous sialic acid as a carbon and energy source by scavenging it from the environment [4, 16]. Once transported into the bacterial cell, sialic acid is sequentially converted to fructose-6-P by enzymatic machinery coded for by genes clustered together on the chromosome (nan operon) (Figure 1A and 1B). In silico analysis of the genome of the GBS strain 515Ia revealed a putative nan operon (Figure 1A), which contained among the clustered genes an N-acetylmuraminic lyase (nanA, sal0044), a putative transcriptional regulator (nanR, sal0047), an N-acetylmannosamine-6-P epimerase (nanE, sal0038), a predicted triripartite transporter formed by 2 integral membrane components (sal0040 and sal0041), and a sugar-binding unit (sal0039). The putative nan operon was present in all available GBS genomes, suggesting an essential biological relevance of the activity mediated by the factors encoded by the genes clustered in this area. We also identified a putative CcpA binding site located on the promoter region upstream of sal0038 (Figure 1A), indicating that this operon may be negatively regulated by glucose.

Exogenous Sialic Acid Induces SAL0039 Expression
In order to catabolize exogenous sialic acid, bacteria must use specific transporters to import the monomeric form of this sugar [7]. The first stage of this process requires the recognition of sialic acid by bacterial surface sugar-binding ligands. We proposed that SAL0039, the putative sialic acid binding unit, may play a key role in sequestering this sugar from the environment and making it accessible for transport. The assumed protein product of sal0039 gene is a peptide of 438 amino acids containing a Sec-dependent signal sequence (residues 1–31) and an N-terminal domain (residues 58–348) belonging to the bacterial extracellular solute-binding family 1 (PF01547) (Figure 2A). We initially observed by Western blotting analysis that in glucose rich medium (THB) the protein was not expressed at any growth conditions (data not shown). Based on this, we hypothesized that the amount of glucose in THB (approximately 10 mM) could be responsible for the inhibition of SAL0039 expression. Therefore, we performed protein expression analysis of bacteria grown in a previously described peptone-based CM [11] to which sialic acid (Neu5Ac), glucose, human alpha-acid glycoprotein (AGP), or pooled human serum were added (Figure 2B). Western blotting analysis on total extracts derived from bacteria grown in CM alone revealed that the SAL0039 protein was constitutively expressed, as indicated by a clear band running at the expected molecular weight (approximately 43 kD) (Figure 2B). The addition of 10 mM glucose completely abolished SAL0039 expression, confirming our hypothesis. On the contrary, in the presence of 2 mM Neu5Ac, we observed a dramatic increase of SAL0039 expression. Bacteria grown in CM containing 2 mM AGP (a serum glycoprotein containing a terminal α-2,3–linked sialic acid residue) did not significantly alter SAL0039 expression in respect to the basal level. This was somewhat expected because the inability of GBS to cleave AGP terminal-linked sialic acid results in the lack of free sialic acid in the medium [17]. On the other hand, when GBS was grown in the presence of 30% human serum, SAL0039 expression was slightly downregulated with respect to CM alone. These data were in line with semiquantitative reverse-transcription PCR analysis of RNA transcript levels of bacteria grown in THB vs human blood, showing a dramatic decrease of sal0039 and sal_0044 gene transcripts (Supplementary Figure 1). Comparative global gene expression analysis of GBS grown in high glucose conditions confirmed the negative regulation of the genes clustered in the nan operon (B. Di Palo et al, unpublished data).

Deletion of sal0039 Results in GBS Impaired Growth in the Presence of Neu5Ac
We have previously shown that GBS is able to gain carbon and energy from complex alpha-glucans found in the
Although there is evidence that GBS is able to synthesize de novo sialic acid [18], whether it uses environmental sialic acid as a growth source remains unknown. We hypothesized that SAL0039, by contributing to extracellular Neu5Ac uptake, may modulate bacterial growth. To prove this hypothesis, we generated an isogenic \(515\Delta sal0039\) mutant strain by in-frame deletion of the gene (see Material and Methods). As shown in Figure 3A, the \(515\Delta sal0039\) strain had a reduced capacity to grow in CM supplemented with 2 mM Neu5Ac compared with the \(515\)wt strain. As expected, deletion of \(sal0039\) did not affect the ability of GBS to grow in the presence of glucose (Figure 3A). In agreement with the fact that GBS lacks the ability to scavenge sialic acid from polysaccharide side chains of glycoproteins [17], no growth differences were observed when AGP was added to the CM. To confirm that the GBS growth phenotype in the presence of sialic acid is due to a real consumption of the sugar, we measured the levels of residual Neu5Ac in the medium at the end of the growth phase. As shown in Figure 3B, the \(515\)wt strain consumed nearly all of the substrate by the end of the growth in CM supplemented with 2 mM Neu5Ac. On the contrary, the levels of Neu5Ac did not change after incubation with the \(515\Delta sal0039\) mutant strain, indicating that the deletion of the gene completely abolished the ability of GBS to import the sugar from the growth medium. These data suggest that SAL0039 is essential for GBS intracellular transport of free environment [11]. Although there is evidence that GBS is able to synthesize de novo sialic acid [18], whether it uses environmental sialic acid as a growth source remains unknown. We hypothesized that SAL0039, by contributing to extracellular Neu5Ac uptake, may modulate bacterial growth. To prove this hypothesis, we generated an isogenic \(515\Delta sal0039\) mutant strain by in-frame deletion of the gene (see Material and Methods). As shown in Figure 3A, the \(515\Delta sal0039\) strain had a reduced capacity to grow in CM supplemented with 2 mM Neu5Ac compared with the \(515\)wt strain. As expected, deletion of \(sal0039\) did not affect the ability of GBS to grow in the presence of glucose (Figure 3A). In agreement with the fact that GBS lacks the ability to scavenge sialic acid from polysaccharide side chains of glycoproteins [17], no growth differences were observed when AGP was added to the CM. To confirm that the GBS growth phenotype in the presence of sialic acid is due to a real consumption of the sugar, we measured the levels of residual Neu5Ac in the medium at the end of the growth phase. As shown in Figure 3B, the \(515\)wt strain consumed nearly all of the substrate by the end of the growth in CM supplemented with 2 mM Neu5Ac. On the contrary, the levels of Neu5Ac did not change after incubation with the \(515\Delta sal0039\) mutant strain, indicating that the deletion of the gene completely abolished the ability of GBS to import the sugar from the growth medium. These data suggest that SAL0039 is essential for GBS intracellular transport of free...
Neu5Ac and that this mechanism may contribute to bacterial growth in glucose-limited conditions.

Increasing Concentrations of Sialic Acid Revert Glucose Negative Regulation of SAL0039

Physiological concentrations of glucose at mucosal interfaces range from millimolar concentrations (ie, vaginal secretions) to almost undetectable levels (ie, human airway secretions) [19–22]. On the other hand, free sialic acid is rarely found in mucosal secretions because it is mainly linked to host proteins and lipids [16]. However, a number of pathological conditions, including viral infections and inflammatory diseases, are associated with increased levels of free sialic acid, which is mainly released by both host and microbial neuraminidase activity [4, 23]. We hypothesized that under these conditions the local availability of this sugar to residential GBS may affect SAL0039 expression. We performed Western blotting analysis of GBS extracts from bacteria grown in CM containing 3 mM glucose to which increasing concentrations of N-acetylneuraminic acid (Neu5Ac) were added (range, 0.01–1 mM). A, Sugar-free CM; B, CM plus 3 mM glucose; C, As in B plus 0.01 mM Neu5Ac; D, As in B plus 0.1 mM Neu5Ac; E, As in B plus 1 mM Neu5Ac. A typical blot, out of 3 experiments performed with identical results, is shown.

Figure 2. Sialic acid regulates SAL0039 expression. A, Modular organization of the SAL0039 protein from the 515 strain. The signal peptide sequence (SP) is shown in dark gray, and the bacterial extracellular solute-binding family 1 (PF01547) domain is shown in light gray. B, Western blotting analysis of SAL0039 expression in the 515 strain grown in sugar-free complex medium (CM) or CM plus different components (3 mM glucose [Glc], 10 mM Glc, 2 mM alpha-acid glycoprotein [AGP], 30% human serum [HS], and 2 mM N-acetylneuraminic acid [Neu5Ac]). Bacterial total extracts were separated by 10% (weight/volume) sodium dodecyl sulfate polyacrylamide gel electrophoresis. Blots were overlaid with a mouse anti-SAL0039 polyclonal antibody and stained with an antismouse horseradish peroxidase-conjugated antibody. A typical blot, out of 3 experiments performed with identical results, is shown.

Figure 3. The capacity of group B streptococcus (GBS) to grow in the presence of N-acetylneuraminic acid (Neu5Ac) depends on SAL0039 expression. A, The graph represents the growth curves relative to GBS 515 wild-type (515wt) strain (solid line) and 515Δsal0039 mutant strain (dashed line) grown in complex medium (CM) alone (lozenges) or with the addition of 2 mM Neu5Ac (circles), 2 mg/mL alpha-acid glycoprotein (AGP) (squares) or 2 mM glucose (triangles). A typical experiment, out of 4 performed giving identical results, is shown. B, Consumption of sialic acid by GBS in CM was determined using the thiobarbituric acid assay as described in the Materials and Methods. White column indicates the level of sialic acid in CM supplemented with 2 mM Neu5Ac; black and gray columns are relative to the consumption of sialic acid in CM supplemented with 2 mM Neu5Ac by 515wt strain and 515Δsal0039 mutant strain, respectively. The data are the mean of 3 independent experiments ± standard deviation. KO, knock-out; WT, wild-type.

Figure 4. Sialic acid reverts glucose inhibition of SAL0039 expression. Western blotting analysis of group B streptococcus extracts from bacteria grown in (CM) containing 3 mM glucose to which increasing concentrations of N-acetylneuraminic acid (Neu5Ac) were added (range, 0.01–1 mM). A, Sugar-free CM; B, CM plus 3 mM glucose; C, As in B plus 0.01 mM Neu5Ac; D, As in B plus 0.1 mM Neu5Ac; E, As in B plus 1 mM Neu5Ac. A typical blot, out of 3 experiments performed with identical results, is shown.
SAL0039 Contributes to In Vivo Infection of Mucosal Surfaces

In vitro evidence that sialic acid exploitation by GBS depends on SAL0039 led us to hypothesize a role for this protein during in vivo mucosal colonization. This was investigated by using an in vivo mouse model of intranasal and intravaginal GBS infection in which neuraminidase-mediated release of sialic acid was simulated by adding free Neu5Ac. The 515wt and the knockout 515Δsal0039 strains were tested for their capacity to infect mice in the absence or in the presence of exogenous Neu5Ac (dose, 1 mg/mouse). Briefly, animals were intranasally challenged with bacteria at day 0 and at day 1, and sialic acid was administered at the site of infection at day 2. Colony-forming units from mucosal and lung washes were counted at day 3. For intravaginal infections, a single bacterial challenge was done at day 1. As summarized in Figure 5A and 5B, mucosal administration of Neu5Ac strongly increased the number of wild-type bacteria recovered in the nose with respect to PBS-treated animals (over 28-fold increase; geometric mean, 7128 vs 247 CFUs/mL; \(P = .045\)). Notably, the addition of Neu5Ac did not affect the ability of the 515Δsal0039 mutant strain to both colonize the nasopharynx (geometric mean, 260 CFUs/mL; \(P = .84\) vs 515wt + PBS and \(P = .53\) vs 515Δsal0039 + PBS groups) and reside in lungs (undetectable; identical to both 515wt + PBS and 515Δsal0039 + PBS groups). As expected, 515Δsal0039 bacterial loads in PBS-treated animals were similar to those obtained with 515wt strain both in the nose (geometric mean, 544 vs 247 CFUs/mL; \(P = .15\)) and in the lungs (bacteria undetectable with both strains; \(P = .88\)).

A similar approach was applied to a model of intravaginal infection (Figure 5C). Vaginal colonization by the 515wt strain was poor, with 4 of 10 animals having undetectable bacteria. The addition of Neu5Ac resulted in an increased number of infected animals (9 of 10), with a >30-fold increase of the geometric mean of the number of CFUs per milliliter (649 vs 20 CFUs/mL; \(P = .01\)). As for the nasopharynx, the addition of sialic acid was ineffective in promoting vaginal persistence of the 515Δsal0039 mutant strain (geometric mean, 32 vs 20 CFUs/mL; \(P = .73\)). Infection of mice with a complemented 515Δsal0039 strain significantly restored the wild-type phenotype (geometric mean, 298 vs 32 CFUs/mL; \(P = .04\)). These results provide evidence for the importance of SAL0039-mediated transport of sialic acid during colonization of mucosal surfaces.

DISCUSSION

In this study, we report that GBS can use exogenous sialic acid as an energy and carbon resource. We demonstrated that this capacity depends on the presence of a specific transport system that allows the intracellular translocation of environmental Neu5Ac. In addition, deletion of the sal0039 gene results in the complete inability to import extracellular Neu5Ac, suggesting that this might be the only mechanism used by GBS. This finding is in agreement with recent work showing that sialic acid transport in Streptococcus pneumoniae is totally dependent on SP1681-3, an ABC transporter with high homology to the GBS Neu5Ac tripartite transporter investigated in our study [24]. Apart from glucose that is present at approximately 3 mM concentration in vaginal secretions, the vaginal environment lacks easily metabolized sugars. Therefore bacteria inhabiting this niche must acquire nutrients from complex carbohydrates, such as glycogen, which is released by host cells, or heavily glycosylated chains of mucus proteins. Group B streptococcus has been reported to produce a surface-exposed type 1 pullulanase enzyme with specific activity on glycogen and others alpha-glucans [11]. Because no neuraminidase activity has been
described for GBS, we propose that the possibility for this bacterium to metabolize exogenous sialic acid should reside on the presence of other sialidase-producing microbial organisms sharing the same niche [16, 25–27]. The fact that the addition of exogenous sialic acid, which mimicks endogenous sialidase activity, increases GBS capacity to colonize mucosal surfaces supports our hypothesis that the availability of the free form of this sugar during the initial phases of streptococcal colonization could be determinant to a successful settlement. Because the 515Δsal0039 mutant strain shows an impaired capacity to infect mice in the presence of free sialic acid, it can be argued that this phenotype is the result of a reduced availability of nutrients rather than a difference in capsule sialylation. Indeed 515wt and isogenic 515Δsal0039 mutant strains grown in the presence of exogenous Neu5Ac equally survive in a whole human blood assay (data not shown), suggesting that the contribution of environmental sialic acid in capsule sialylation should be minimal. Moreover, in vitro adhesion assays (Supplementary Figure 2) comparing 515wt and 515Δsal0039 grown in THB or in CM supplemented with 2 mM Neu5Ac indicated that bacterial adherence to cell monolayers is independent of SAL0039 expression. These data postulate that the increased number of bacteria recovered from animals treated with sialic acid and infected with 515wt strain (compared with 515Δsal0039) is mainly due to augmented bacterial growth. In the last years, human influenza virus infections have been definitively associated with secondary bacterial pneumonia, defining a central role for viral neuraminidase during bacterial superinfections [28, 29]. Indeed the removal of terminal sialic acid residues from mucosal surfaces unmasks cell-associated ligands, leading to an increased bacterial adhesiveness. A further, but not less important, factor related to viral infections is the availability of free sialic acid that can in turn be used for supporting the growth and biofilm formation of bacterial pathogens [30]. The failure of exogenous Neu5Ac to sustain colonization of the mouse upper respiratory tract by the 515Δsal0039 mutant strain definitively supports the hypothesis of a similar scenario in GBS.

In conclusion, we think that our findings, which unravel the mechanism by which GBS imports sialic acid and how this process may be crucial to an efficient colonization of mucosal surfaces, may contribute to both a better definition of GBS pathogenesis and to the development of novel therapeutic strategies aimed at combating GBS infections.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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**Potential conflicts of interest.** All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

**References**


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