Evolutionary inactivation of a sialidase in group B Streptococcus

Masaya Yamaguchi1,2, Yujiro Hirose1,3, Masanobu Nakata1, Satoshi Uchiyama3, Yuka Yamaguchi2, Kana Goto1, Tomoko Sumitomo3, Amanda L. Lewis4, Shigetada Kawabata1 & Victor Nizet5,6

Group B Streptococcus (GBS) is a leading cause of bacterial sepsis and meningitis in newborns. GBS possesses a protein with homology to the pneumococcal virulence factor, NanA, which has neuraminidase (sialidase) activity and promotes blood-brain barrier penetration. However, phylogenetic sequence and enzymatic analyses indicate the GBS NanA ortholog has lost sialidase function—and for this distinction we designate the gene and encoded protein nonA/NonA. Here we analyze NonA function in GBS pathogenesis, and through heterologous expression of active pneumococcal NanA in GBS, potential costs of maintaining sialidase function. GBS wild-type and ΔnonA strains lack sialidase activity, but forced expression of pneumococcal NanA in GBS induced degradation of the terminal sialic acid on its exopolysaccharide capsule. Deletion of nonA did not change GBS-whole blood survival or brain microvascular cell invasion. However, forced expression of pneumococcal NanA in GBS removed terminal sialic acid residues from the bacterial capsule, restricting bacterial proliferation in human blood and in vivo upon mouse infection. GBS expressing pneumococcal NanA had increased invasion of human brain microvascular endothelial cells. Thus, we hypothesize that nonA lost enzyme activity allowing the preservation of an effective survival factor, the sialylated exopolysaccharide capsule.

Streptococcus agalactiae (Group B Streptococcus, GBS) is a Gram-positive bacterial pathogen that is a leading cause of sepsis, pneumonia, and meningitis during neonatal period and up to the first 90 days of life1,2. Each of the 10 different GBS capsular polysaccharide types3, though possessing different repeating subunits, share a terminal α-2-3-linked sialic acid (N-acetylated neuraminic acid, Neu5Ac motif), which is identical to a sugar epitope cupping many surface glycans on all mammalian cells4. Humans in particular express just the terminal α-2-3-linked Neu5Ac since they have lost the gene required to synthesize the alternative sialic acid, N-glycoly neuraminic acid (Neu5Gc) present in other mammals including primates5. The GBS sialylated capsule mimics a common presentation of Neu5Ac in the α-2-3-linkage, which contributes to evasion of the host immune system and promoting bacterial survival in vivo1. GBS capsular sialylation interferences with the host complement system to block C3b deposition and limit C5a deposition6,7, and inhibits neutrophil activation through interaction with inhibitory sialic acid-binding immunoglobulin-like lectin-9 (Siglec-9)8. The in vivo significance of these findings was corroborated in mice with and without Siglec-E, the closest homolog of human Siglec-9, which interacts with GBS in a sialic acid-dependent manner, triggering protein tyrosine phosphatase, SHP-1, recruitment to its intracellular domain and suppressing myeloid cell inflammatory responses9.

Streptococcus pneumoniae (pneumococcus) is a related Gram-positive pathogen and a major cause of pneumonia, sepsis, and meningitis9,10. Most severe S. pneumoniae diseases occur in children younger than 2 years and adults older than 65 years. The polysaccharide capsule of S. pneumoniae confers the antigenicity utilized to classify S. pneumoniae into at least 97 serotypes11. In contrast to GBS, no S. pneumoniae strains express sialic acid in its capsular polysaccharide. Instead, the bacterium expresses three sialic acid-cleaving enzymes or sialidases, NanA, NanB, and NanC11,12. The nanA and nanB genes are located in the same operon and detected in almost all clinical isolates, whereas the nanC gene is present in approximately half (51%) of isolates12. While the

1Department of Oral and Molecular Microbiology, Osaka University Graduate School of Dentistry, Suita, Osaka, Japan. 2Department of Pediatrics School of Medicine, University of California San Diego, La Jolla, CA, USA. 3Department of Urology, Nagoya University Graduate School of Medicine, Nagoya, Aichi, Japan. 4Department of Molecular Microbiology and Ob/Gyn, Washington University School of Medicine, St. Louis, Missouri, USA. 5Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California San Diego, La Jolla, CA, USA. 6Rady Children’s Hospital, San Diego, CA, USA. Correspondence and requests for materials should be addressed to M.Y. (email: yamaguchi@dent.osaka-u.ac.jp)
molecular functions of NanB and NanC in the pathogenesis are unclear, NanA has been identified as a multifunctional protein contributing to pneumococcal virulence\(^1\). NanA is a cell-wall-anchored protein and works as an invasin into human brain microvascular endothelial cells (hBMEC) through its LamG superfamily domain\(^1\). An isogenic \(S. pneumoniae\) \(ΔnanA\) mutant strain showed >90% reduction in adhesion and invasion efficiency compared to its parent strain; complementation of NanA expression on a plasmid vector restored the adherence/invasion phenotype. Furthermore, heterologous expression of NanA in \(Lactococcus lactis\) conferred an adhesion and invasion frequency ~10-fold greater than empty-vector-transformed control\(^1\). The NanA LamG domain induces inflammatory cytokine production from the brain endothelial cells, and the resulting cell activation promotes pneumococcal internalization\(^1\). In addition, desialylation of leukocyte cell surfaces by NanA resulted in MAP kinase phosphorylation and NF-κB activation through unmasking of Siglec-5\(^1\).

Here, we identify through homology searching a putative ortholog of pneumococcal NanA that is present in GBS strains. The biological consequences for GBS of possessing a potential sialidase enzyme, while simultaneously expressing a sialylated capsule as an essential virulence determinant, were initially unclear. Our bioinformatics analysis suggested that, unlike pneumococcal NanA, the GBS orthologue has lost the LamG domain and cell wall-anchoring motif, and that there was a nonsense mutation in this gene in some GBS strains. Codon-based selection analysis indicated that pneumococcal \(nanA\) was under stronger negative selection than \(nonA\). We find that the GBS strains do not possess neuraminidase activity, and for this distinction we designate the gene and encoded protein \(nonA\)/\(NonA\). In contrast to earlier published findings with pneumococcal NanA mutants\(^13,14,16\), targeted deletion of the \(nonA\) gene in GBS did not alter resistance to human whole blood killing, brain microvascular endothelial cell invasion, or animal virulence. However, forced expression of active pneumococcal NanA in the GBS \(ΔnonA\) mutant removed terminal sialic acid from the GBS polysaccharide capsule, reducing GBS survival in whole blood, while promoting GBS invasion of brain microvascular endothelial cells. Taken together, our results strongly suggest that the loss of function as a sialidase in GBS NonA in contemporary GBS strains allowed the organism to preserve the selective advantage of sialylated capsule.

**Results**

**Evolutionary analysis of a GBS \(nanA\) ortholog.** We performed a bioinformatics analysis on the \(nanA\) gene, an ortholog of pneumococcal \(nanA\) (\(SAK_{RS09520}\) or \(SAK_{1891}\)), annotated in the published genome of GBS strain A909. Amino acid sequence alignment analysis showed that \(SAK_{RS09520}\) contains a sialidase domain but lacks the conserved lectin-like domain LamG and cell wall anchoring motifs present in pneumococcal NanA (Fig. 1A). GBS NonA shared 58% amino acid sequence identity with pneumococcal NanA across the sialidase domain, with lesser degrees of sequence identity with pneumococcal NanB and NanC (27–28% amino acid sequence identities) (Supplementary Table 1). Next, tBLASTn analysis revealed that a subset of species in the genus \(Streptococcus\) contains \(nanA\) orthologs and a phylogenetic analysis was performed using orthologous bacterial \(nanA\) sequences. Both Bayesian- and maximum likelihood phylogenetic analyses of these orthologs revealed similar patterns of genetic classification with high posterior probabilities or bootstrap values (Fig. 1B, Supplementary Fig. 1 and Supplementary Table 2). The sialidase genes of Gram-positive and rod-shaped bacteria, \(Erysipelothrix rh梳opathiae\), \(Clostridium perfringens\), and \(Virgibacillus sp.\), were used to root, since with the exception of homologous genes from other streptococci, these genes exhibited the highest similarity with pneumococcal \(nanA\). \(E. rh梳opathiae\) and \(C. perfringens\) are known to produce active sialidases\(^18–20\). Both trees indicate that the \(nanA\) ortholog genes of \(S. mitis\) and \(S. pseudopneumoniae\) diverged from each other, having shared a common ancestor. Of note, \(nanA\) genes of \(S. pneumoniae\) strains CGSP14 and NT-110_58 were distinct from those of other pneumococcal strains. The phylogenetic analysis revealed that the GBS \(nonA\) represents a single lineage in a cluster otherwise composed of \(S. iniae\), which is a pathogen of fish and occasional nosocomial infections in humans\(^21\). NonA of both \(S. iniae\) and GBS lack an LPXTG motif, which is conserved in the NanA proteins of other streptococcus species. All streptococcal sialidases except GBS NonA possess the LamG domain (Fig. 1A and Supplementary Fig. 2). Further analysis of the genome database indicates that five GBS strains (GX026, SA20-06, 2-22, 138spar, and 138P) carry a \(nanA\) gene containing a nonsense mutation (Supplementary Table 3). In addition, we measured bacterial sialidase activities using streptococcal type strains and clinical isolates (Supplementary Fig. 3). Type strains of \(S. oralis\), \(S. intermedius\), and \(S. pseudopneumoniae\) showed positive sialidase activities. In contrast, the sialidase activity of \(S. mitis\), GBS, and \(S. iniae\) strains was always below the detection limit. Previously, Killian et al. reported that 100% of 17 \(S. pneumoniae\) and 3 \(S. pseudopneumoniae\) strains and 69% of 54 \(S. mitis\) strains showed positive sialidase activity\(^22\). Some \(S. mitis\) strains appear to have reduced the genome sizes and may have lost virulence-associated factors including NanA in a reductive evolutionary process\(^23,24\). Thus, it is likely that \(S. mitis\) strains exhibit a diversity of sialidase activity. Furthermore, the result of an ancestral reconstruction technique suggests the possibility that sialidase activity was lost in the \(nonA\) lineage rather than gained in the \(nanA\) lineage (Fig. 2). Together these results suggest that streptococcal \(nanA\) orthologs diverged into two major groups, one consisting of \(S. mitis\), \(S. intermedius\) and \(S. pneumoniae\), and the other consisting of GBS and the \(S. iniae\) group. In the \(S. iniae\)/GBS group \(NonA\) appears to have lost its functional role.

To examine the relationship of pneumococcal \(nanA\), \(nanB\), \(nanC\), and GBS \(nonA\), a phylogenetic analysis was performed using the genes. Bayesian- and maximum likelihood phylogenetic analyses of the genes revealed similar patterns of genetic classification with high posterior probabilities or bootstrap values (Fig. 3 and Supplementary Fig. 4). The pneumococcal \(nanB/nanC\) were well separated from pneumococcal \(nanA\) or GBS \(nonA\). We performed an additional evolutionary analysis on \(nanA\), \(nonA\), \(nanB\), \(nanC\), \(bgaA\), and \(strH\) genes. BgaA and \(strH\), another pneumococcal exoglycosidases, remove galactose that is \(β1-4\) linked to \(N\)-acetylglucosamine, and \(N\)-acetylglucosamine that is \(β1-3\) linked to mannose, respectively\(^13\). Selection analysis through non-synonymous/synonymous ratio calculations by Fixed Effects Likelihood (FEL) and Fast, Unconstrained Bayesian AppRoximation (FUBAR) analyses suggested similar results. There were more codons evolving under negative selection in the \(nanA\) genes of \(S. pneumoniae\) strains (Table 1, Supplementary Table 4).
Figure 1. Phylogenetic analysis of nanA orthologs. (A) Schematic illustration of domains in *S. pneumoniae* NanA and GBS NonA. NonA lacks LamG domain and LPXTG motif conserved in NanA. (B) Bayesian phylogenetic tree of the nanA and nonA genes. The information on bacterial strains is listed in Supplementary Table 2. Strains with identical sequences are listed on the same branch. Percentage of posterior probabilities is shown near the nodes. The scale bar indicates nucleotide substitutions per site. *S. pneumoniae* nanA and GBS nonA genes are shaded in blue and red, respectively.
and Supplementary Figs 5–10). In contrast, fewer codons evolving under negative selection were detected in the

\textit{nonA} genes of GBS as well as the \textit{nanB} and \textit{nanC} genes. Similar results were obtained with the \textit{bgaA} and \textit{strH} genes, indicating that \textit{nanA} is under strong selective pressure. On the other hand, there were no or very few codons that appear to be evolving under positive selection in these genes. We conducted a likelihood ratio test to investigate whether pneumococcal \textit{nanA} and GBS \textit{nonA} genes have the same distribution of substitution rates across sites (Table 2). The distributions of substitution rates indicate no significant differences in between pneumococcal \textit{nanA} and GBS \textit{nonA} genes. However, there was a significant difference in selective regimes (dN/dS

\textbf{Figure 2. Ancestral state reconstructions based on the Bayesian phylogenetic tree.} Parsimony reconstruction using Mesquite for active or inactive sialidase phenotypes is shown as white or black lines, respectively. Gray lines indicate missing values.

inactive
| active
| missing value
Figure 3. Bayesian phylogenetic tree of nanA, nanB, nanC, and GBS nonA genes. Percentage of posterior probabilities is shown near the nodes. Strains with identical sequences are listed on the same branch. The scale bar indicates nucleotide substitutions per site. Blue shows pneumococcal nanA and green is nanB or nanC. GBS nonA is shown as red.

Table 1. Evolutionary analyses of nanA, nonA, nanB, nanC, bgaA, and strH genes. Evolutionary analysis was performed using Bayesian inference of aligned nanA, nonA, nanB, nanC, bgaA, or strH sequences from complete genomes of S. pneumoniae or S. agalactiae, with two rate FEL in the HyPhy software package. The dN/dS means ratio of non-synonymous changes to synonymous changes in overall analyzed genes. Individual codons with a statistically significant signature were also calculated and are expressed as a percentage of the total number of codons used in the analysis.
The expression of the complemented ΔnonA brain endothelial cells.

pared to that of other strains; however, invasion of the complemented GBS ΔnonA strains showed similar histogram patterns and did not interact with the FITC-labeled ECA. On the other hand, the ECA binding level inversely reflects the level of sialylation on the GBS capsule. GBS WT and FITC-labeled terminal sialic acid moiety on the GBS capsular polysaccharide repeating unit by flow cytometry with sialidase activity could be detected with heterologous expression of the pneumococcal enzyme.

ΔWT nor ΔnonA GBS strains showed sialidase activity associated the bacterial cells or culture supernatants, but sialidase activity could be detected with heterologous expression of the pneumococcal enzyme.

We next investigated whether heterologous expression of a functional sialidase (NanA) would degrade the terminal sialic acid moiety on the GBS capsular polysaccharide repeating unit by flow cytometry with FITC-labeled Erythrina cristagalli agglutinin (ECA; Fig. 4B). ECA binds to terminal (unsialylated) galactose and the ECA binding level inversely reflects the level of sialylation on the GBS capsule. GBS WT and ΔnonA mutant strains showed similar histogram patterns and did not interact with the FITC-labeled ECA. On the other hand, the complemented GBS ΔnonA[pNanA] strain showed substantially higher fluorescence intensity when incubated with FITC-labeled ECA as compared to the strains incubated without FITC-labeled ECA. These results indicated that the GBS WT strain possessed no sialidase activity and the forced expression of the active sialidase in GBS could have the effect of degrading its own terminal sialic acid, a known immune evasion virulence factor of the pathogen with anti-complement, anti-phagocytic, and immunosuppressive properties.

Forced expression of NanA in GBS degrades terminal sialic acids of its capsule. To investigate the role of NonA in bacterial pathogenesis, we constructed an isogenic GBS ΔnonA mutant strain and then complemented the ΔnonA strain with the functional pneumococcal NanA as described in the Methods section. The expression of the nonA gene in a GBS wild-type (WT) strain was higher than that of the well-characterized cyIE gene encoding the GBS β-hemolysin/cytolysin (Supplementary Fig. 11). Sialidase activities of GBS WT, ΔnonA, and ΔnonA[pNanA] strains were determined using a fluorometric sialidase assay (Fig. 4A). Neither the WT nor ΔnonA GBS strains showed sialidase activity associated the bacterial cells or culture supernatants, but sialidase activity could be detected with heterologous expression of the pneumococcal enzyme.

NonA does not contribute to GBS invasion into hBMECs. To examine the role of GBS NonA compared to the previously established role of pneumococcal NanA in the invasion of blood-brain barrier endothelium, we performed adherence/invasion assay using human brain microvascular endothelial cells (hBMECs) (Fig. 5). To quantify bacterial invasion, hBMECs were incubated with GBS strains for 1 hour (h) and further incubated for 1 h in medium containing antibiotics. WT GBS and the ΔnonA mutant strains showed no significant change of NanA would be deleterious in S. pneumoniae. In fact, pneumococcal NanA is a multifunctional protein, that promotes bloodstream survival and penetration of host endothelial cell barriers system. In contrast to pneumococcal NanA, the GBS NonA does not appear to be under strong selective pressure, which supports our hypothesis that NonA no longer functions in GBS.

| Tests                                      | LR  | DF | P-value |
|--------------------------------------------|-----|----|---------|
| The distributions                          | 14.853 | 10 | 0.137   |
| Selective regimes (dN/dS and proportions)  | 9.602 | 2  | 0.008   |
| Selection strength (dN/dS)                 | −0.020 | 1  | 1.000   |
| The proportions of codons under selection  | 8.120 | 1  | 0.004   |

Table 2. Comparing codon selection between nanA and nonA genes. Comparing codon selection was performed using Bayesian inference of aligned nanA or nonA sequences, and distribution comparison tests in the HyPhy software package. LR; Likelihood ratio. DF; degrees of freedom.

Discussion

S. pneumoniae contains three sialidases, NanA, NanB, and NanC. NanB works as a virulence factor in pneumococcal infection and NanC catalyzes intermediate metabolic compounds which acts as sialidase inhibitor. The nanC gene was reported to significantly associate with clinical isolates from invasive diseases. Our evolutionary analysis indicated that ~15% of the codons in the pneumococcal nanA gene evolved under negative selection, while few codons of nanB and nanC evolved in positive or negative selection. The results suggest that selection pressures exist such that the key enzymatic functions of nanA may not change. In contrast to the
Figure 4. NanA degrades terminal sialic acid displayed on GBS polysaccharide capsule. (A) Sialidase activities of GBS cells and culture supernatant. After 2 h incubation at 37 °C, fluorescence of sialidase-degraded substrate was measured with excitation and emission wavelengths of 350 and 460 nm, respectively. Data are presented as the mean of sextuplets samples. S.E. values are represented by vertical lines. The sensitivity is 0.3 mU/mL. (B) FITC-labeled ECA binding to live GBS. Red line and blue histogram represents data for bacterial strains incubated without or with ECA, respectively.
conservations on nanA in S. pneumoniae, the GBS NanA homologue (NonA) appears to have lost its sialidase activity. Sialyltransferases are highly conserved in GBS strains of each serotype when compared to other glycosyltransferase genes in a same operon, and the difference in genetic diversity support a hypothesis that sialic acid is critical for GBS survival in the human host. Our results showed that restoration of an active sialidase function inhibited GBS survival in human blood ex vivo and mouse blood in vivo. Therefore, sialidase activity would be deleterious to the fitness of GBS, and GBS nonA appears to be a non-functional gene.

We recently reported a similar relationship between bacterial capsule and glycosidase in another pathogenic streptococci, group A Streptococcus (GAS, Streptococcus pyogenes). Almost all serotypes of GAS express a hyaluronan exopolysaccharide capsule and contain an inactivated version of the hyaluronidase (HylA) with a single nucleotide mutation resulting in Asp to Val substitution at amino acid position 199. However, serotype M4 strains express an active HylA, while lacking hyaluronan capsule biosynthesis operon. The operon was predicted to represent a more recent evolutionary acquisition in most serotypes. Although hyaluronan capsule is a major GAS virulence factor, heterologous expression studies to generate partial encapsulation of M4 wild-type strain and full encapsulation of an isogenic mutant ΔhylA strain did not increase virulence. In this human bacterial pathogen, the conflicts between polysaccharide capsule and glycosidase would exert conflicting selective pressures, and resulted in mutual exclusivity. In the present work, we find a similar mutual exclusivity between sialidase activity and the GBS polysaccharide capsule.

It is widely thought that pathogenic microbes may explain some human polymorphisms. Sialylated pathogens can dampen the immune response through interaction with Siglecs, and this molecular mimicry is considered to be one of the primary forces in the rapid evolution of human Siglecs. For example, Siglec-13 and -17 may have been genetically eliminated during hominid evolution, because of interactions with pathogenic bacteria, including GBS, that cause invasive infections. In addition, Siglec-14 and -5 expressed on neutrophils and monocytes appear to have evolved to provide a balanced response to pathogens and infants with Siglec-14 deficiency were the most prone to GBS immune subversion. Thus, there exists a multifaceted interaction between pathogen and human evolution at the molecular level. The synergy of evolutionary bioinformatics and functional analysis may help to investigate the interplay between pathogen and host within an evolutionary framework and to identify new genetically stable therapeutic targets within pathogens and/or their human hosts.

**Methods**

**Phylogenetic and evolutionary analysis.** Phylogenetic and evolutionary analyses were performed as previously described with minor modifications. Homologues of nanA were searched for using tBLASTn of NCBI BLAST. Sequences from complete genomes with e-values <2 × 10^{-85} and >40% query coverage were selected for phylogenetic tree analysis. The sequences were aligned using MAFFT with FFT-NS-i strategy and edited using by Jalview. Regions coding sialidase domain were used for further phylogenetic analysis. Edited sequences were aligned again using MAFFT with L-INS-i strategy. The best-fitting codon evolutionary models for maximum likelihood and Bayesian phylogenetic trees were determined by RAxML ver. 8.1.20 and MrBayes ver. 3.2.5, sampling 10^6 generations with a confirmation that the standard deviation of split frequencies was <0.01. Phylogenetic trees were drawn by FigTree ver. 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/) based on calculated data.

Tests for evolutionary analysis were performed on aligned common codon sequences of nanA, nonA, nanB, nanC, bgaA, or strH genes. Complete identical sequences were excluded. Whole gene non-synonymous/synonymous (dN/dS) ratio calculations, as well as statistical tests for negative or positive selection for individual codons,
were performed using two-rate Fixed Effects Likelihood (FEL) and Fast Unconstrained Bayesian AppRoximation (FUBAR) in the HyPhy software package. Comparing codon selection between \( \text{nanA} \) and \( \text{nonA} \) genes was performed using LR tests in the HyPhy.

Ancestral states for bacterial sialidases were reconstructed in Mesquite version 3.04 using a parsimony model with characters treated as unordered. The reconstruction was performed on the phylogenetic tree generated by MrBayes. States of active or inactive sialidase were assigned “0” or “1” for each taxon. Unavailable data were coded as missing.

**Bacterial strains and cell lines.** Streptococcal strains listed in Supplementary Table 5 were cultured in Todd-Hewitt broth (BD Biosciences) supplemented with or without 0.2% yeast extract (BD Biosciences) (THY or TH medium) at 37°C. *Streptococcus pseudopneumoniae* ATCC BAA-960 (also called as SK1069 or CCUG...
bacterial association.

The rate of bacterial association was also calculated by dividing the number of bacterial invasion by the number of original inoculums. The invasion of 100 adherent cells was analyzed with a CyFlow SL flow cytometer.

After 20 h post-infection, blood aliquots were collected from mice just after general euthanasia. The samples of brain/meninges were collected following perfusion with PBS. Bacterial counts in blood and brain homogenates were determined by plating serial dilutions. Bacterial counts in brain were corrected for differences in each brain weight.

**Construction of mutant strain.** The construction of in-frame deletion mutants was conducted using a temperature-sensitive shuttle vector, pSET4s, as reported previously.48,49. During the course of construction, a merodiploid strain was created after the first allelic replacement and then resolved to possess either mutant or wild type alleles after the second allelic replacement. To minimize the effect of secondary mutations and epigenetic changes that may have arisen during mutagenesis, a clone possessing the wild-type allele was used as a wild-type strain. Both the wild-type and an in-frame deletion mutant strain arose from the same merodiploid ancestor. The correct in-frame deletion of genes was confirmed by site-specific PCR using purified chromosomal DNA. To create NonA and NanA-swapped GBS strain, ΔnonA[pNanA], pNanA plasmid was introduced respectively into GBS ΔnonA strain by electroporation.51 pNanA was constructed by ligating nanA gene from S. pneumoniae strain D39 into pDESTerm plasmid.49

**Sialidase activity assay.** Sialidase activities of bacterial cells and supernatants were determined by Neuraminidase assay kit (abcam). Streptococcal strains were grown to the mid-log phase (OD_600 = 0.4–0.5) and centrifuged. To prepare bacterial cell fraction, the bacterial pellet was washed by PBS and resuspended in PBS. The supernatant was used as a supernatant fraction. The samples were incubated for 2 hours at 37°C and fluorescence intensity was measured at Ex/Em = 350 nm/460 nm.

**Real-time reverse transcription-PCR (RT-PCR) assay.** Total RNA of GBS strains grown to the exponential phase (OD_600 = 0.5) was isolated with RNeasy mini kit and RNase-Free DNase Set (Qiagen). Then, cDNA was synthesized with Transcriptor First Strand cDNA Synthesis Kit (Roche). Real-time RT-PCR analysis was conducted using StepOnePlus Real-Time PCR system (Thermo Fisher Scientific) and KAPA SYBR Fast qPCR Kit (KAPA Biosystems). Data for gyrA were used as internal control. Primers are listed in Supplementary Table 6.

**ECA-binding assay.** ECA-binding assay was performed as previously described.51 GBS strains were grown to the mid-log phase and resuspended in PBS to adjust OD_600 to 0.1. The bacteria were incubated on ice with FITC-conjugated Erythrina cristagalli agglutinin (ECA; Vector Laboratories, CA) at 10 μg/mL for 30 min. Then, bacterial cells were washed and resuspended in PBS. The ECA-binding activities on the surface of live bacterial cells were analyzed with a CyFlow SL flow cytometer.

**hBMEC association and invasion assay.** The bacterial association to and invasion of hBMEC were quantified with minor modifications as described previously.52–54. GBS strains were grown to mid-log phase (OD_600 = 0.5) and resuspended in PBS (OD_600 = 0.1). hBMECs were seeded at 2 × 10⁵ cells per well in RPMI1640 supplemented with 10% FBS in 24-well plates 1 d prior to bacterial infection. In each well, ~2.0 × 10⁶ CFU of bacteria was added to infect with ~2.0 × 10⁵ hBMECs at a multiplicity of infection (MOI) of 10 in a final volume of 500 μL, and the plate was centrifuged at 1600 rpm for 5 min to initiate their contact. To determine bacterial adhesion, the infected cells were incubated for 1 h, washed three times with PBS, and harvested with a trypsin and 0.025% Triton X-100 solution. The number of bacterial association was quantified by serial dilution plating. To examine bacterial invasion, hBMECs were washed following 1 h-incubation, and 500 μL of media containing 100 μg/mL of gentamicin was added and cells were incubated for an additional 1 h. The cells were washed and lysed, and the number of bacterial invasion was quantified. The bacterial association or invasion rate was calculated by dividing the number of bacterial association/invasion by the number of original inoculums. The invasion rate of bacterial association was also calculated by dividing the number of bacterial invasion by the number of bacterial association.

**Blood bactericidal assay.** A blood bactericidal assay was performed as previously described.52,55,56. Blood was obtained via venipuncture from healthy donors. It was performed under written informed consent according to a protocol approved by the institutional review boards of Osaka University Graduate School of Dentistry. The GBS strains grown to the mid-log phase were washed and resuspended in PBS, and OD_600 was adjusted to 0.1. Bacterial cells (10^11) were combined with fresh human blood (190 μl), and then the mixture was incubated at 37°C in 5% CO₂ for 1, 2, and 3 hours. Viable cell counts were determined by plating diluted samples onto THY agar. Growth index was calculated as the number of CFU at the specified time point/number of CFU in the initial inoculum.

**Mice infection assay.** All mouse experiments were conducted in accordance with animal protocols approved by the Animal Care and Use Committees at Osaka University Graduate School of Dentistry (24-025-2). CD-1 (ICR: IGS) mice (6 weeks, female; Oriental) were infected with 3.5 × 10⁶ CFU of GBS via the tail vein. After 20 h post-infection, blood aliquots were collected from mice just after general euthanasia. The samples of brain/meninges were collected following perfusion with PBS. Bacterial counts in blood and brain homogenates were determined by plating serial dilutions. Bacterial counts in brain were corrected for differences in each brain weight.
Statistical analysis. Statistical analysis of in vitro and in vivo experiments was performed using a nonparametric analysis, Mann–Whitney U test. The tests were carried out with Graph Pad prism version 6.0e (GraphPad Software, Inc.). In evolutionary analysis, P < 0.1 was regarded as a significant difference as well as HyPhy default setting.

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**Author Contributions**

M.Y., S.K. and VN. designed the study. M.Y. and Y.Y. performed bioinformatics analysis. M.Y. and S.U. performed enzyme assay. M.N. and S.U. performed real-time RT-PCR assay. M.Y. performed adhesion and invasion assay, and bactericidal assay. M.Y., Y.H. and K.G. performed mouse infection assay. M.N., M.Y., S.U. and A.L.L. constructed mutant strains. M.Y., M.N., T.S., A.L.L., S.K. and V.N. wrote the manuscript.

**Additional Information**

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