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Attenuation of *Streptococcus suis* virulence by the alteration of bacterial surface architecture

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NeuB, a sialic acid synthase catalyzes the last committed step of the *de novo* biosynthetic pathway of sialic acid, a major element of bacterial surface structure. Here we report a functional NeuB homologue of *Streptococcus suis*, a zoonotic agent, and systematically address its molecular and immunological role in bacterial virulence. Disruption of *neuB* led to thinner capsules and more susceptibility to pH, and *cps2B* inactivation resulted in complete absence of capsular polysaccharides. These two mutants both exhibited increased adhesion and invasion to Hep-2 cells and improved sensibility to phagocytosis. Not only do they retain the capability of inducing the release of host pro-inflammatory cytokines, but also result in the faster secretion of IL-8. Easier cleaning up of the mutant strains in whole blood is consistent with virulence attenuation seen with experimental infections of both mice and SPF-piglets. Therefore we concluded that altered architecture of *S. suis* surface attenuates its virulence.

Sialic acids (Sia) are a group of acidic sugars with a common nine-carbon backbone that is widespread on the surface of nearly all vertebrate cells[1,2]. N-acetylneuraminic acid (Neu5Ac, seen in **Figure 1**) is the most prevalent type of Sia and plays a series of roles in multiple biological processes including physiology, evolution and immune response[3–5]. Neu5Ac has been found to be decorated on the surface structure of a growing number of bacterial pathogens, including Group B *Streptococcus* (GBS)[1,6]. This type of molecular mimicry appears to act as an effective strategy used by pathogens to survive in the infected host environments[7] and can result in serious host immunological dysfunctions[2,8,9]. As a known virulence determinant in GBS, surface capsular polysaccharides (CPS), have been confirmed to display a terminal N-acetylneuraminic acid (Neu5Ac), a common type of Sia[2]. These Neu5Ac residues may sometimes be followed by O-acetylation modification[10], which has posed significant implications for GBS pathogenesis and immunogenicity[1,11]. Given that bacterial surfaces decorated with glycol-conjugates can function in multiple biological processes (like cell-to-cell signaling and cell-to-host communication[12]), it seems reasonable that modification and/or alteration of the surface structure of the human symbiont bacterium *Bacteroides fragilis* affects its localization during host-symbiont mutualism[13]. To the best our knowledge, bacteria have developed two alternative strategies to fulfill the requirement of sialic acid-decoration of bacterial surface structure: *de novo* endogenous biosynthesis (**Figure 1A**), and scavenging of host Sias[2]. Therefore elucidation of machinery for bacterial sialic acid's metabolism is important for better understanding Sia-dependent virulence mechanism present in the major human pathogens.

Streptococcus suis serotype 2 (*S. suis* 2, SS2) is a Gram-positive swine pathogen[14], and also regarded as an increasingly-important opportunistic human agent[15]. Two types of human SS2 infections in Asian countries like China have ever been recorded: sporadic cases[16], and relatively-big scale outbreaks[17]. Unlike P1/7, an international SS2 virulent strain, the epidemic strain of 05ZYH33, features a unique 89K pathogenicity island (PAI)[18–20]. Additionally, we have identified many genes essential for bacterial virulence[21–24] and immunogenicity[25,26] from this epidemic strain 05ZYH33 originally isolated in China. In general agreement with an observation with GBS (**Figure 1A**), we also noticed an operon with *neuB* included on the chromosome of 05ZYH33 strain, suggesting the presence of Sia biosynthesis pathway[20,27]. NeuB, the *neuB* protein product, catalyzes the last committed step of bacterial sialic acid biosynthesis, via the condensation of phosphoenolpyruvate (PEP) and N-acetylmannosamine (ManNAc) to form Neu5Ac (**Figure 1 B&C**)[28]. Our particular interest in *neuB* gene of Chinese virulent SS2 strain 05ZYH33 is due to following three reasons:1) this strain causes epidemics of human

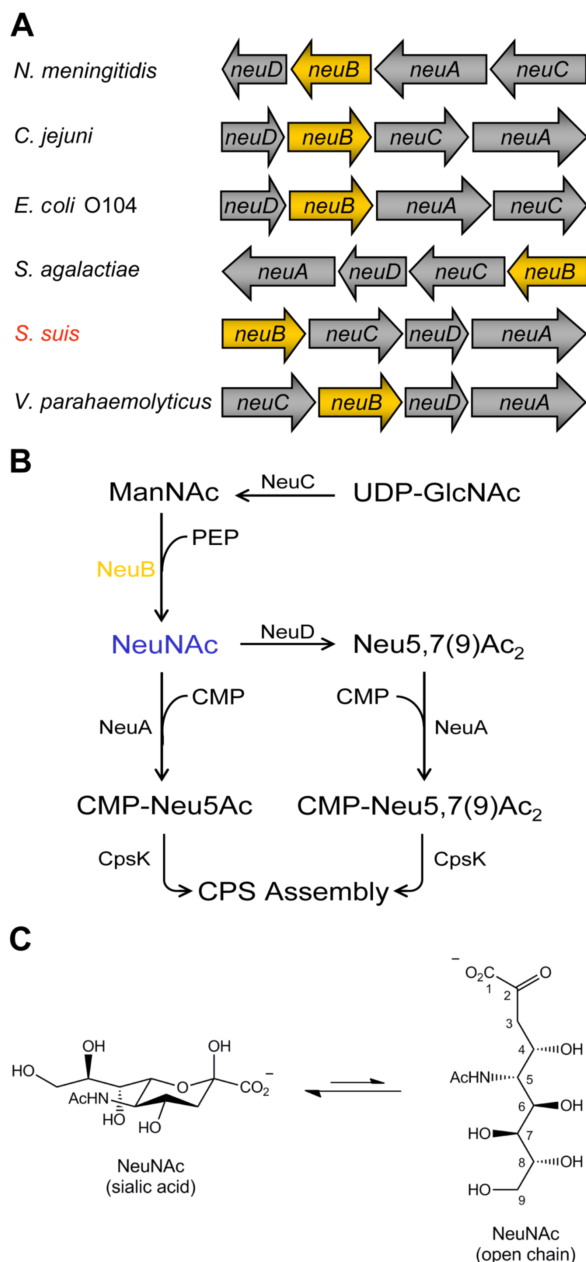

Figure 1 | Genetic organization of gene clusters and current working model for bacterial sialic acid biosynthetic pathway and subsequent metabolism. (A) Genetic organization of the gene loci involved in *de novo* synthesis and utilization of bacterial sialic acids. *neuB* that encodes sialic acid synthetase is highlighted in golden-yellow. *S. suis* is indicated in red. The abbreviations are as follows: *N. meningitidis* for *Neisseria meningitidis*; *C. jejuni* for *Campylobacter jejuni*; *E. coli* for *Escherichia coli*; *S. agalactiae* for *Streptococcus agalactiae*; *S. suis* for *Streptococcus suis* and *V. parahaemolyticus* for *Vibrio parahaemolyticus.* (B) Working model for the biosynthetic route and metabolism of bacterial sialic acids. NeuB, sialic acid synthase is shown in golden-yellow, and NeuNAc (also abbreviated as Neu5Ac, N-acetylneuraminic acid), a prevalent form of sialic acids is indicated in blue. NeuA terms as sialic acid O-acetylesterase, NeuC refers to UDP-GlcNAc 2-epimerase, NeuD is defined as sialic acid O-acetyltransferase[12] and CpsK is an oligosaccharide α-2,3-sialyltransferase[10,50]. All other abbreviations are listed as follows: ManNAc, *N*-acetylmannosamine; GlcNAc, *N*-acetylglucosamine; UDP- GlcNAc, Uridine diphosphate N-acetylglucosamine; Neu5,7(9)Ac_2, N-acetyl-7 or 9-O-acetylneuraminic acid; CMP-Neu5,7(9)Ac_2, CMP-Neu5Ac, Cytidine monophosphate N-acetylneuraminic acid; Cytidine monophosphate N-acetyl-7 or 9-O-acetylneuraminic acid; CPS, Capsule polysaccharide. (C) Chemical structures of NeuNAc, the leading type of sialic acids in two existing states.

SS2 infection clinically featuring streptococcal toxic shock syndrome[17], 2) NeuB determines the final rate-limiting step of bacterial Neu5Ac biosynthesis, and 3) earlier reported preliminary evidence[29] indicated that *neuB* might be correlated with virulence of S735 strain. Although Wilson *et al.*[29] unexpectedly observed that the predicted *neuB* locus in S735 strain of *S. suis* 2 is associated with bacterial virulence during the development of signature-tagged mutagenesis, the detailed functional and/or structural aspects regarding NeuB sialic acid synthase and its role in bacterial pathogenesis is still lacking.

In this paper, we aim to close above knowledge gap. Thereafter we generated two null mutants (Δ*neuB* and Δ*cps2B*) with the background of an epidemic strain 05ZYH33, and systematically addressed genetic/immunological aspects in the process of bacterial interplay with hosts. Also, we are the first to attribute *S. suis* NeuB homologue to a functional member of the sialic acid synthase family by structural modeling.

Results

Discovery and characterization of *S. suis* NeuB homologue. The *cps* operon of *S. suis* 05ZYH33 strain consists of 20 putative open reading frames (ORFs) that range from 05SSU0562 to 05SSU0581 (not shown). Among them, four loci (05SSU0577, 05SSU0578, 05SSU0579, and 05SSU0580) are separately identified as *neuB*, *neuC*, *neuD* and *neuA* (**Figure 1A**), whose protein products are involved in the pathway of sialic acid metabolism (**Figure 1B**). Multiple sequence alignments show that *S. suis* NeuB homologue is highly similar to the well-studied NeuB proteins from both *E. coli* or *N. meningitidis* (**Figure 2**). Of particular note, 15 critical active sites revealed by structural and functional dissections of the *N. meningitidis* NeuB[28] were found to be extremely conserved in *E. coli* and *S. suis* (**Figure 2**). Although *S. suis* NeuB exhibited only 35.5% identity to that of *N. meningitidis,* the protein tertiary structures of both NeuB enzymes are similar (**Figure 3A&B**). Indeed, structural superposition analyses further confirm that the modeled structure of *S. suis* NeuB matches with the architecture of *N. meningitidis* NeuB (**Figure 3**). The pocket formed by the putative active sites was clearly seen in *S. suis* NeuB (Supplemental **Figure 2**). Additionally, our RT-PCR results demonstrated that *S. suis neuB* can be transcribed at the mRNA level (**not shown**). Given the above combined data, we anticipated that *S. suis neuB* homologue encodes a functional sialic acid synthase.

Alteration of surface capsular structure by deletion of *neuB* from *S. suis*. Using the method of homologous recombination, we aimed to construct two null mutants of *S. suis* 05ZYH33 (Δ*neuB* and Δ*cps2B*). The mutants of interest (in which a double-crossover event occurred) were confirmed by series of combined evidence that include colony PCR, RT-PCR, Southern blot, etc. (Supplemental **Figure 1**). As we anticipated, in contrast with the wild type strain 05ZYH33, the both mutants (Δ*neuB* and Δ*cps2B*) failed to agglutinate with the serotype 2-specific serum (**Figure 4A**), implying that disruption/alteration of surface capsular structure is due to functional impairments of either *neuB* or *cps2B*. Subsequent analyses using transmission electron microscopy revealed that the capsule thickness of Δ*neuB* (50∼70 nm) is significantly thinner than that of its parental strain 05ZYH33 (110∼130 nm) (**Figure 4A**). The capsule of the Δ*neuB* mutant seemed to be much more compact relative to that of wild type. This defection in the bacterial capsule was restored by functional complementation of a plasmid-borne *neuB* gene (**Figure 4B**). Thiobarbituric acid assays show that sialic acid content is significantly decreased in the Δ*neuB* mutant in comparison with its parental strain (**Figure 5A**). It is reasonable to conclude that the inactivation of *neuB* that encodes sialic acid synthase definitely disturbed the *de novo* biosynthesis pathway of sialic acid. However, we did not note any other changed microbiological phenotypes, such as the altered bacterial

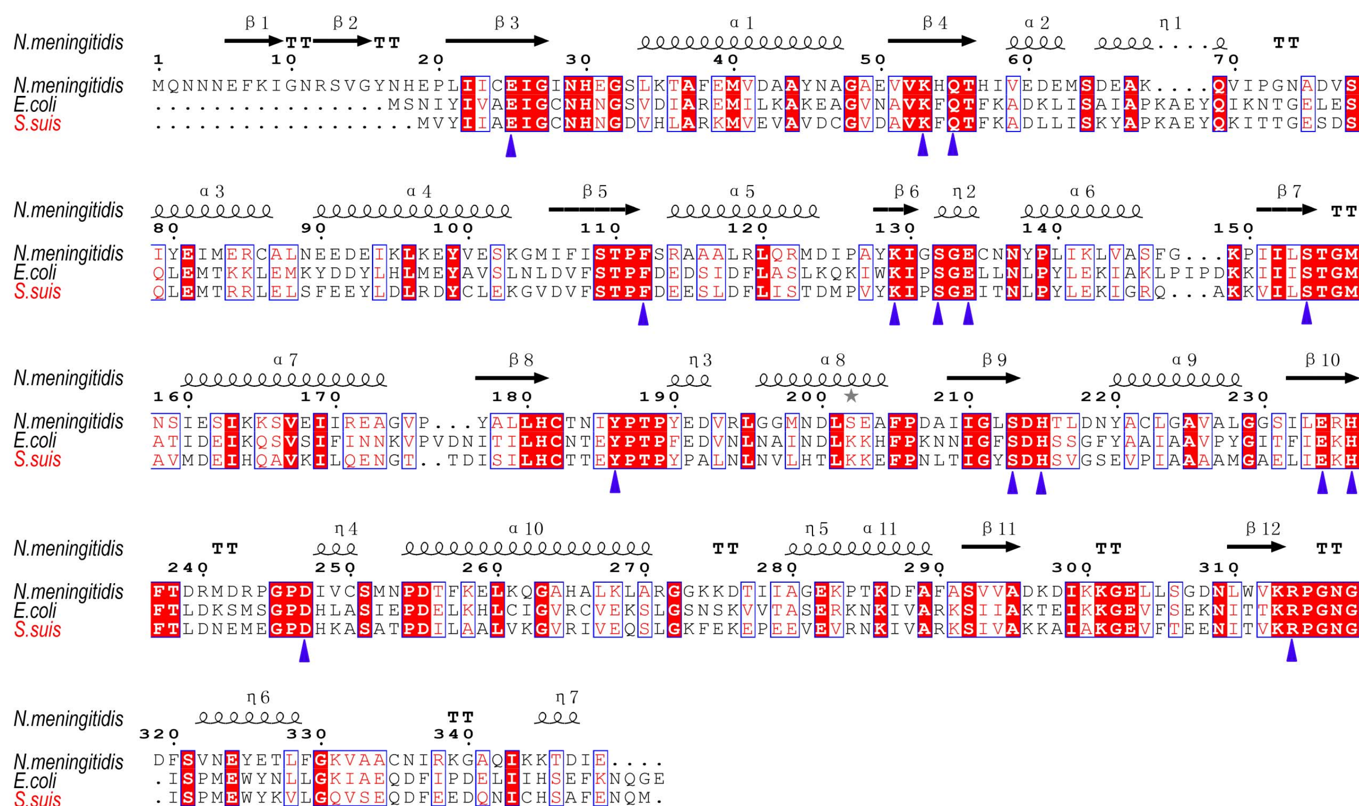

Figure 2 | Multiple sequence alignments of *S. suis* NeuB homologue with two known NeuB proteins from *E. coli* and *N. meningitides*. The amino acid sequences of NeuB proteins used here are separately collected from *S. suis* 05ZYH33 (YP_001197944), *E. coli* K12 (NC_010498), and *N. meningitides* (AAA20477). *S. suis* was indicated in red, and *N. meningitides* is an abbreviation for *Neisseria meningitides*. Identical residues are indicated with white letters on a red background, similar residues are red letters on white, varied residues are in black letters, and dots represent gaps. The predicted secondary structure of the NeuB protein is shown on top. α: α-helix; β: β-sheet; T: β-turns/coils. Fifteen critical residues (such as E25, 53K, 55Q, etc.) visualized in crystal structure of *N. meningitidis* NeuB protein for its enzymatic activity and/or Mn^{2+} binding[28] are found to be extremely conserved and thereby highlighted with blue triangles.

Figure 3 | Modeled structure of *S. suis* NeuB and configuration of its active sites. (A) Ribbon representation of *N. meningitidis* NeuB structure. (B) The modeled three-dimension structure of *S. suis* NeuB. (C) Structure superposition of *S. suis* NeuB with the counterpart of *N. meningitidis*. The structure of *N. meningitidis* NeuB (PDB: 1XUZ) is in golden, whereas that of *S. suis* NeuB (with 35.5% identity to that of *N. meningitidis* NeuB) is in purple. Designations: N, N-terminus; C, C-terminus.

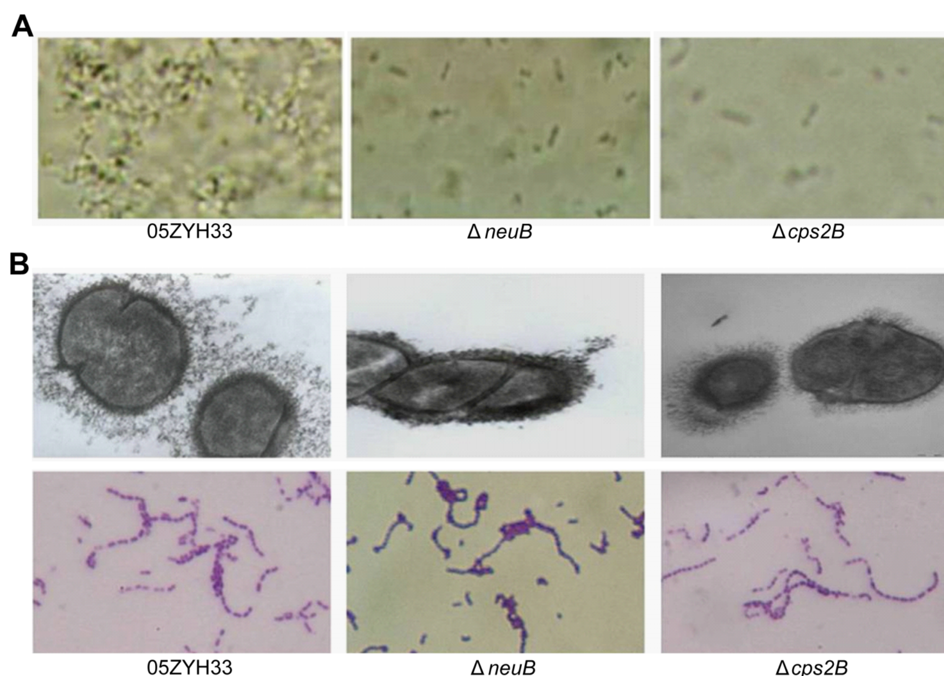

Figure 4 | Altered microbiological properties in the two mutants (Δ*neuB* & Δ*cps2B*). (A) Antiserum agglutination assays of the two mutants (Δ*neuB* & Δ*cps2B*) in comparison with the wild type 05ZYH33 in 20 seconds. (B) Comparative microscopic analyses of mutants plus its parental strain. Transmission electron microscopy (TEM)-based analyses (×50000) are shown in top panel, whereas the Gram-staining tests under light microscopy (×1000) are presented in bottom.

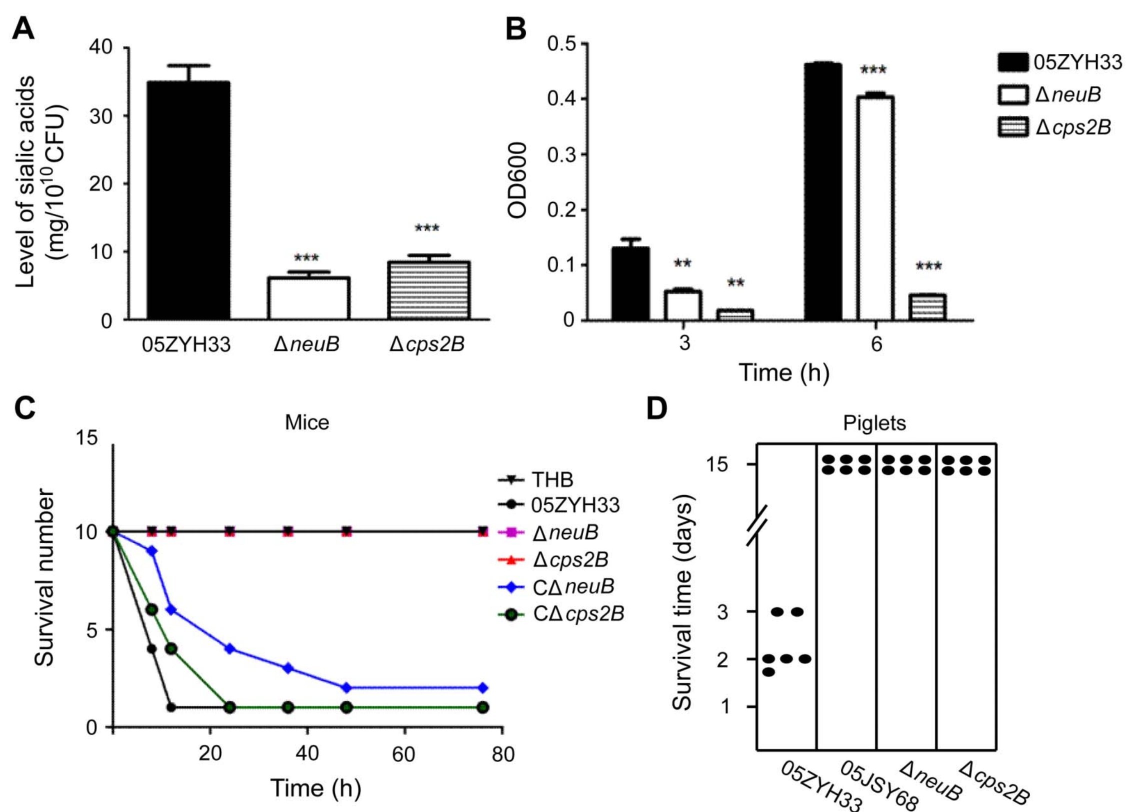

Figure 5 | Virulence attenuation of the epidemic Chinese *S. suis* strain, 05ZYH33 by altered structure of Sia-capped surface. (A) Decreased level of bacterial sialic acids in the mutants (Δ*neuB* & Δ*cps2*). The asterisk indicates that the concentration of sialic acid of the mutants was significantly decreased, at a P value of <0.05. (B) The growth of 05ZYH33, Δ*neuB*, and △*cps2B* in pH5.0. The asterisk indicates that the growth values of the mutants were significantly lower than that of the wild-type strain, at a P value of <0.05. Comparative analyses of bacterial virulence of the mutants with the wild type strain using the models of mice (in **Panel C**) and piglets (in **Panel D**).

growth curves or increased/decreased sensitivity to the H_2O_2 environment (**Figure 5B**).

Requirement of *neuB* for *S. suis* virulence. To further prove the putative role of NeuB and/or CPS in *S. suis* pathogenesis, we utilized two different experimental models (SPF-mice as well as its natural host, mini-piglet). In general consistency with our earlier observations[21,22,25], nine out of ten SPF-mice died within 12 hours of being intra-peritoneally inoculated with wild type virulent strain 05ZYH33 (positive control) while the remaining mouse developed serious symptoms within 24 hours. In contrast, ten mice infected with the Δ*cps2B* mutant strain (an avirulent strain as the negative control), survived without any obvious symptoms until the end of this experiment (14 days after infection). Of particular note, all ten mice challenged with the Δ*neuB* mutant survived within 24 hours, with only three showing slight symptoms (such as swollen eyes) after 12 hours of infection and recovering within 48 hours (**Figure 5C**). Furthermore, all the mice injected with the complemented strains (CΔ*neuB* and CΔ*cps2B*) died within 2 days, in general agreement with the observations with wild type strains (**Figure 5C**). Intriguingly, a new report appeared in this month, suggesting that another member NeuC with the function in sialic acid synthesis pathway is associated with S. suis virulence in mouse model[51].

Piglet infection experiments showed that most of the typical disease symptoms (high fever, limping, swollen joints, shivering, central nervous system failure, and respiratory failure within 24 hours) could be observed in all six piglets inoculated with the wild type strain, and all piglets died during the course of the experiment. In contrast, the piglets inoculated with mutant strains Δ*cps2B*, Δ*neuB* or avirulent strain 05JSY68 did not exhibit any apparent clinical symptoms and survived untill the end of the experiment (**Figure 5D**). Postmortem assays suggested that 05ZYH33 can be isolated from the central nervous system (CNS), blood, kidney, heart, liver, lung, spleen, joints, and tonsils. Small numbers of the mutant strains could be recovered from the tonsils and joints but were never recovered from the CNS, blood, kidney, liver, lung, joints, heart or spleen (not shown). Given the accumulated data above, we concluded that the disruption of *neuB* and/or *cps2B* gene of 05ZYH33, a Chinese epidemic strain of *S. suis* 2 altered the surface capsule structure, and in turn attenuated bacterial virulence.

Contributions of Sia-capped capsular surface to protection of *S. suis* against host killing. To gain preliminary insights into virulence attenuation by impairment of Sia-capped surface structure, we performed a series of immunological assays. In the whole blood killing test, we observed that the wild type strain 05ZYH33 survived with the human whole blood, while the mutant strain (Δ*neuB* and/or Δ*cps2B*) rapidly died, and reduced to 6%–8% of its original inoculation amount (**Figure 6A**). It implied that Sia-capped surface structure confers bacterial resistance to whole blood killing in the infected host environment. Surprisingly, we found that an improved efficiency of mutants (Δ*neuB* and/or Δ*cps2B*) in both adherence and invasion into Hep-2 and HUVEC cells (**Figure 6B&C**). In general consistency with earlier observations of Segura and coworkers[30], the levels of phagocytosis in the mutant strains (Δ*neuB* and/or Δ*cps2B*) by Raw264.7 murine macrophages were significantly higher than those of their parental strain 05ZYH33 (**Figure 7A**), indicating encapsulated surface structure is involved in resistance to macrophage-mediated phagocytosis.

Intriguingly, although the invasion capabilities of the mutants (Δ*neuB* and/or Δ*cps2B*) with deficiency in their capsular surface structure were pretty stronger relative to the WT strain, 05ZYH33 (**Figure 7B**), their survival rates in THP-1 cells under the pressure of antibiotic treatment were dramatically decreased (**Figure 7C**). However the viable cells of the WT strain, 05ZYH33 can still be recovered from the lysate of THP-1 cells even after 7 hours of antibiotic treatment (**Figure 7C**). It seems likely that Sia-capped polysaccharide surfaces contribute to the survival of *S. suis* virulent strain 05ZYH33 during the fighting against host THP-1 cells.

Further analyses of cytokine release by THP-1 cells showed that entry of pathogenic *S. suis* isolate 05ZYH33 with well-encapsulated surface structures stimulated the release of IL-6/IL-8 incrementally with a peak being reached around 24 h (**Figure 8A&B**). In particular, the Δ*neuB* and/or Δ*cps2B*-induced cytokines (IL-6 and IL-8) reached peak concentrations at 18 h, faster than 05ZYH33.

Discussion

Although there have been controversies as to the roles of sialic acids in *S. suis* virulence[29,31,32], we report integrative evidence that the component of sialic acids localized on the bacterial cell surface is involved in its pathogenesis of 05ZYH33, an epidemic strain of *S. suis* serotype 2. Given that 1) the interplay between *S. suis* and the upper respiratory tract epithelium is essential for initiation of bacterial infection[33] and 2) the invasion of cerebral endothelial cells forming the blood–brain barrier is likely a primary step in the pathogenesis of meningitis caused by *S. suis*[32], we choose the Hep-2 and HUVEC cell lines to address the physiological contributions of sialic acid to the ability of adherence and invasion by *S. suis* 2. In general agreement with the reports by Benga L, *et al.*[34], we observed that the deletion of *neu*B decreased the content of sialic acid and led to the increase of the adhesion and invasion to host cells. We assume that sialic acid might be not conductive to the bacterial adhesion and invasion, probably due to the altered surface capsular structure caused by decreased sialic acid content. In contrast, it seems that sialic acids of the host cell surface can facilitate adherence of *Brucella* to epithelial cells[35].

Bacterial phagocytosis by monocytes/macrophages should be another important event associated with the *S. suis* pathogenesis. Charland *et al.*[36] had reported that unencapsulated mutants of *S. suis* 2 were more susceptible to phagocytosis by macrophages compared to the parent strain. The mechanisms by which *S. suis* avoids phagocytosis were related to host phosphatidylinositol 3-kinase (PI-3K) pathway[37]. Very recently, Houde *et al.*[38] showed that *S. suis* CPS inhibits phagocytosis through destabilization of lipid microdomains and prevents lactosylceramide-dependent recognition. Given the above combined observations, it can in part explain why the phagocytosis levels of the mutant strains (Δ*neuB* and/or Δ*cps2B*) by Raw264.7 macrophages were significantly higher relative to their parental strain 05ZYH33 (**Figure 7**).

We know sialic acids act as an important component of anti-phagocytic factor for many bacterial species, by inhibiting the activation of the alternative complement pathway[39]. This is why we observe that the absence of sialic acid synthase (NeuB) and CPS2B, decreased bacterial resistance to phagocytosis of murine macrophage cells Raw 264.7 and THP-1. Given the strong stimulatory ability of epidemic Chinese strain ST7 in the production of the massive pro-inflammatory cytokines[40], we attempted to probe the role of sialic acid in its inflammatory response. As we expected, all the tested bacteria could induce high levels of inflammatory cytokine and chemokines. Of note, the levels of interleukin IL-8 production by THP-1 stimulated by Δ*neuB* and/or Δ*cps2B* reached peak concentration faster (18 h) than those produced by the wild type strain (24 h) (**Figure 8**). Because IL-8, is the prototype member of the CXC chemokines that fulfills strong neutrophil chemotactic and activating properties and also contains the ability to induce the chemotaxis of $CD4^+$ and $CD8^+$ T cells, it seems likely that the loss of sialic acids might promote the recruitment of chemokines and increase the immune response of host cells to *S. suis* 2 invasion. It may also be implicated in the immuno-suppression of sialic acids which are involved in the immune recognition and the response of host immune cells to *S. suis* 2. A similar scenario we recently encountered was the functional impairment of the T4SS-like system located in the 89K pathogenicity island of an epidemic strain of *S. suis*, 05ZYH33, which greatly affected the release of pro-inflammatory cytokines of host cells[41].

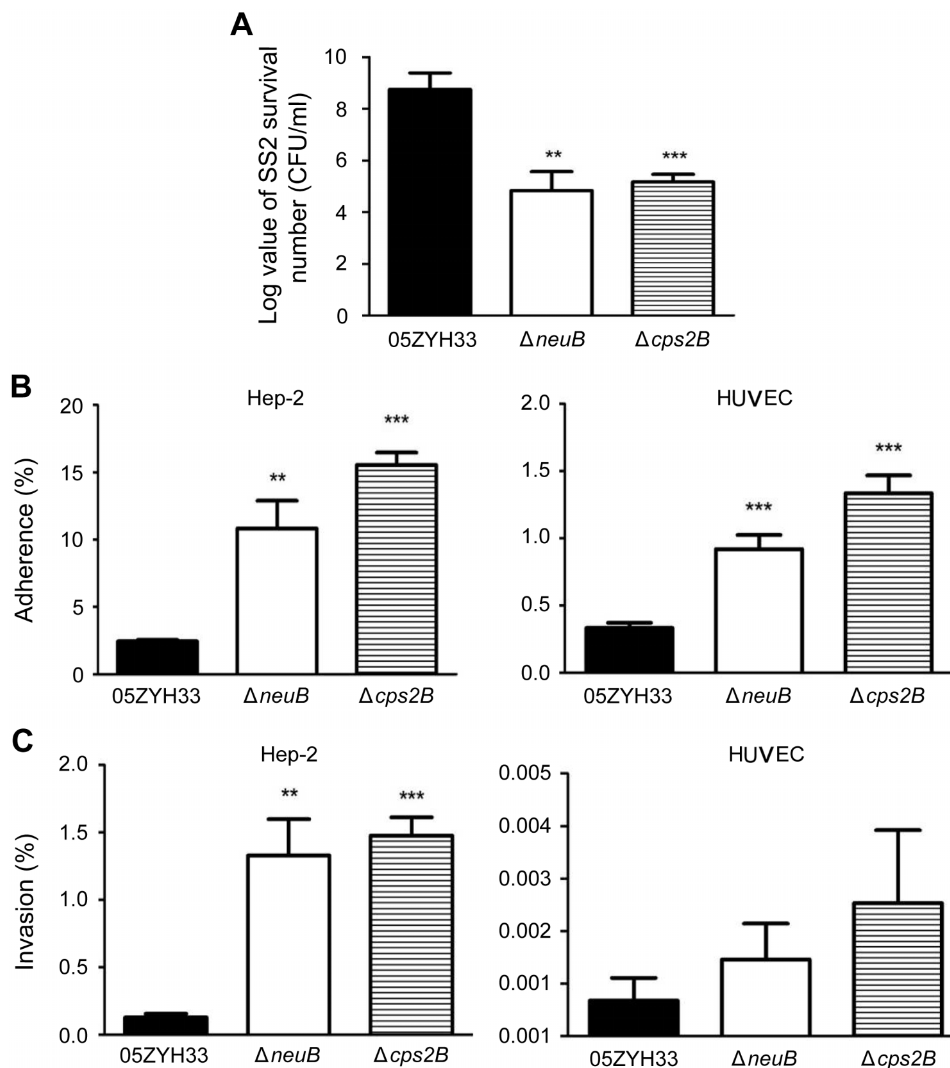

Figure 6 | Resistance to whole blood killing and adherence and invasion ability to Hep-2 and HUVEC. (A) Resistance analyses of *S. suis* strains to human whole blood killing. Following 8 hours of incubation of bacteria (05ZYH33, Δ*neuB* or △*cps2B* at the concentration of 10^6 CFU/ml) with human whole blood, their survival levels were determined by plating. Data shown here are expressed in means ± SD. The asterisk indicated that the survival number of the mutants was significantly lower than that of the wild-type strain, at a P value of <0.05. (B) Enhanced adherence capability of the mutants (Δ*neuB* or △*cps2B*) to Hep-2 and HUVEC cells. Data shown here are means ± SD of intracellular bacteria/ml. The asterisk indicates that the adherence values of the mutants was significantly higher than that of the wild-type strain, at a P value of <0.05. (C) Increased invasion ability of the mutants (Δ*neuB* or Δ*cps2B*) to Hep-2 and HUVEC. Data shown here are means ± SD of intracellular bacteria/ml. The asterisk indicates that the invasion values of the mutants was significantly higher than that of the wild-type strain, at a P value of <0.05.

Methods

Bacterial strains and growth conditions. *E. coli* DH5α was grown in Luria-Bertani Broth[28] liquid medium or plated on LB agar at 37°C. As we earlier reported[17,26,42], all the *S. suis* 2 strains such as 05ZYH33 (Supplemental Table 1) were maintained in Todd-Hewitt broth (THB) liquid and/or agar media. When necessary, appropriate antibiotics were supplemented as follows: spectinomycin (Sigma) for *S. suis* derivatives of *S. suis* 05ZYH33, 100 μg ml^{-1} and ampicillin (Sigma) for *E. coli*, 100 μg ml^{-1}. The two types of cloning vectors, pMD18-T vector (Takara) and pUC18 vector (Promega) were used for either direct DNA sequencing or gene cloning.

Gene inactivation and functional complementation. To generate the two mutants (Δ*neuB* and Δ*cps2B*) of strain 05ZYH33, the entire coding sequences were replaced by homologous recombination with plasmids pUC18::*cps2B* and pUC18::*neuB*, respectively (**Supplemental Figure 1**). In these two knock-out vectors, the target gene of either *neuB* or *cps2B* was designed to be replaced with a spectinomycin resistance (Spc^R) cassette (Supplemental Table 1). Electroporation of *S. suis* 05ZYH33 competent cells were conducted to harvest the possible transformants as we previously established[19,42] with minor change. Subsequently, these colony-purified *spc*^{R} transformants were screened through colony multiplex-PCR assays with series of specific primers (Supplemental Table 2), and the suspected mutants were further verified by Southern blotting (Supplemental **Figure 1**).

For functional complementation, the DNA fragment covering the entire *neuB* (or *cps2B*) coding region plus its putative upstream promoter and downstream regions was cloned into an *E. coli*-*S. suis* shuttle vector, pSET1[42,43], to give plasmid pSET1::*neu*B (or pSET1::*cps2B*). The resulting plasmid was introduced into the Δ*neu*B (or *cps2B*) mutant to make the corresponding complemented strain CΔ*neu*B (or CΔ*cps2B*).

Biochemical and microbial characterization of *S. suis* strains. The thiobarbituric acid assay was applied to determine the capsular sialic acid contents of different *S. suis* strains[44,45]. To evaluate bacterial susceptibility to oxidant, *S. suis* strains were challenged with hydrogen peroxide (H_2O_2) at various level (ranging from 30%, 15%, 7.5%, 3%, 0.3% to 0.03%, vol/vol) for 15 min. The bacterial survival percentage was expressed by dividing the number of CFU at different concentration of H_2O_2 with the initial number of CFU prior to H_2O_2 challenge. For testing bacterial resistance to acids, *S. suis* strains were inoculated in THB liquid media at different pH (7.0, 6.0, 5.0 and 4.0), and the values of their optimal densities at wavelength of 600 (OD600) were recorded at time intervals.

Transmission electron microscopy. Transmission electron microscopy (TEM) was utilized for visualization of effects of *S. suis* morphology by *neuB* deletion[21]. In short, the agar-grown bacterial samples were fixed in 5% glutaraldehyde for 2 h, post-fixed with 2% osmium tetroxide for 2 h, dehydrated in a graded series of acetone washes,

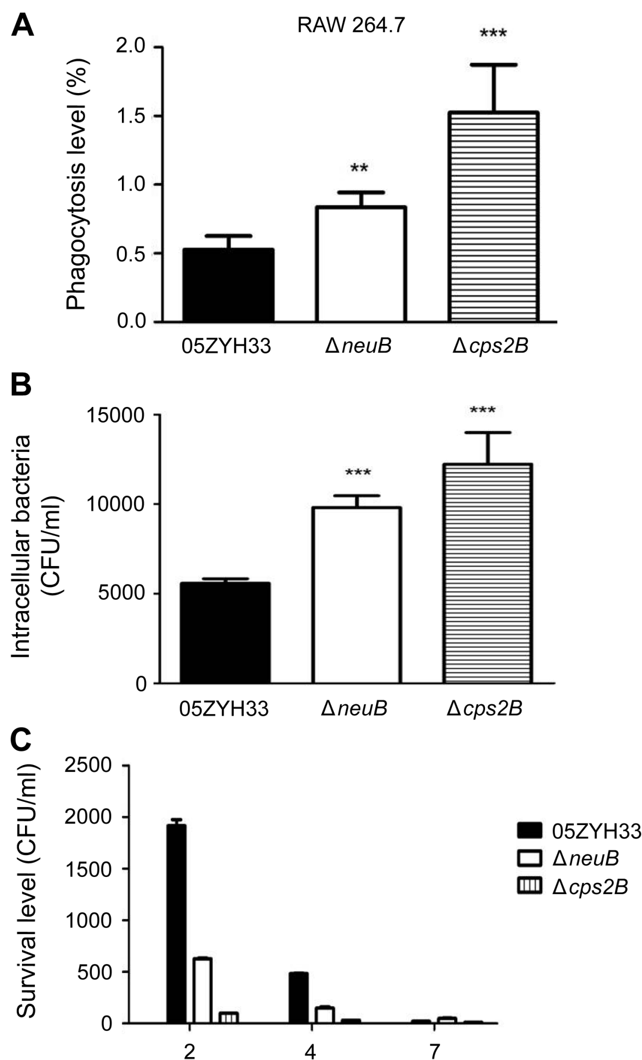

Figure 7 | Phagocytosis analyses of different *S. suis* strains. (A) Phagocytosis of *S. suis* strains by RAW264.7 macrophages. Intracellular bacteria were recovered from cell lysates in 1 h post-infection. The results are expressed as means ± SD of recovered bacteria/ml. The asterisk indicates that the phagocytosis values of the mutants were pretty higher than that of the wild-type strain, at a **P value of <0.05. (B) Intracellular *S. suis* 2 in THP-1. The results are expressed as means ± SD of recovered bacteria/ml. The asterisk indicates that the phagocytosis values of the mutants were significantly higher than that of the wild-type strain, at a ***P value of <0.05. (C) Antibiotic effects on intracellular 05ZYH33 in THP-1 at different time points Data are expressed as means ± SD of recovered bacteria/ml. The survival values of the mutants were significantly lower than that of the wild-type strain, at a P value of <0.05.

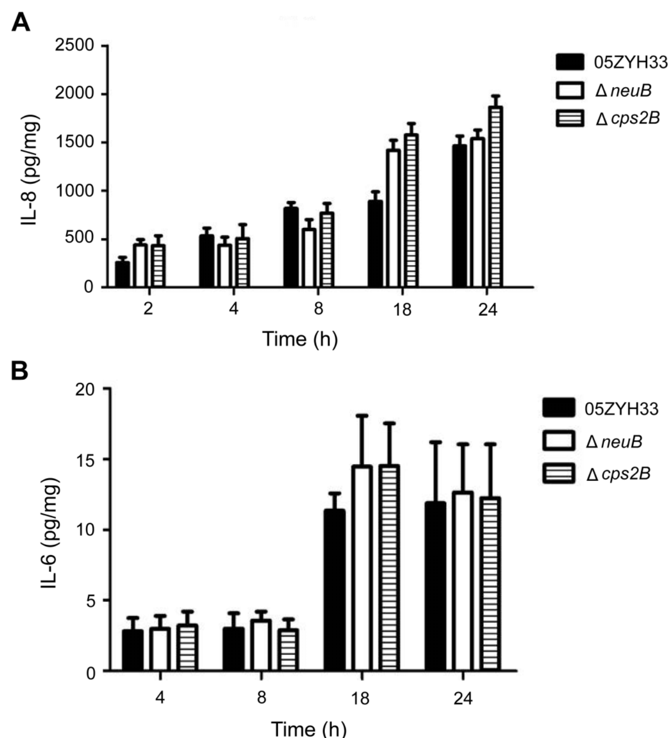

Figure 8 | Cytokine production by THP-1 stimulated with the heat-killed *S. suis* 2. (A) Time course of IL-8 production by THP-1 after the stimulation with heat-killed *S. suis* 2 (B) Time course of IL-6 production by THP-1 after stimulation with the heat-killed *S. suis* 2. Culture supernatants were harvested at different time intervals and assayed for cytokine production by ELISA. Data are expressed as mean ±SD (pg/ml). The P value is less than 0.05.

and embedded in epoxy resin. Thin sections were post-stained with uranyl acetate and lead citrate and then examined with a JEM-1010 TEM (JEOL, Ltd., Tokyo, Japan) at an accelerating voltage of 80 kV.

Infection of experimental animals. Two types of experimental animals (BALB/c mice and SPF-piglets) were employed to test potential relationship between *S. suis* virulence and the altered bacterial surface structure[27]. In infection experiments of mice, 0.5 ml of bacteria cultures (05ZYH33, Δ*neuB*, Δ*cps2B*, CΔ*neuB* and CΔ*cps2B*, 4×10^8 CFU/ ml) was inoculated intra-peritoneally into BALB/c mice (female, 4-week-old). They were divided into four groups, each of which included 10 mice. Mice were clinically monitored for around two weeks, in which deaths were recorded and moribund animals were humanely killed.

The observed pathogenicity of *S. suis* 2 in mice was further verified with the infection model of SPF-piglets, the natural host of *S. suis*. These three-week-old piglets with an average weight of 3.0 kg were randomly allotted to four groups, each consisting of six piglets. These groups of piglets were intravenously challenged via the ear vein with *S. suis* strains (05ZYH33, 05JSY68 and relevant mutants) in the dose of 10^8 CFU/piglet. As we earlier reported[17], clinical observations were performed for 15 days post-inoculation and moribund pigs were humanely sacrificed in time. Samples were taken at necropsy and processed for histological examinations. All animal experiments were approved by Ethics Committee of Research Institute for Medicine of Nanjing Command.

Evaluation of bactericidal activity in human whole blood. An improved assay for whole-blood bactericidal activity[46,47] was conducted to test the possibility that bacterial virulence attenuation is due to decreased ability of multiplication in blood. In brief, inoculum (100μl) containing wild type strain 05ZYH33 and relevant mutant strains at a ratio of 1 : 1 (1×10^6 CFU each) was added to 1 ml of fresh, heparinized whole human blood in sterile glass tube and incubated on an orbital shaker for 3 h at 37°C. Subsequently, 100 μl of sample was removed from the tubes and plated onto THB agar plate in a series of dilution with/without 100 μg/ml of spectinomycin as selective pressure to enumerate viable bacteria. The CFU of the mutants were counted by dilution and colony formation on the selective THB, and the CFUs of 05ZYH33 were the balance of CFUs grown on the selective plate subtracted from that of pure THB. The survival index was calculated as the CFU recovered after the 3 h incubation divided by the CFU in initial inoculum.

Assays for cell adhesion/invasion and intracellular survival. Experiments of cell adhesion and/or invasion were performed with two different cells: one is human laryngeal epithelial cell line Hep-2 (CCTCC GDC004) and the other is human umbilical vein endothelial cells (HUVEC). In these experiments, *S. suis* strains (05ZYH33, Δ*neuB* and Δ*cps2B* strain) were used. As we recently described[21], The percentage of bacterial adherence was expressed as following formula, (CFU on plate/ CFU in original inoculum)×100%.

To quantify the amount of intracellular bacteria, we also conducted an assay for bacterial invasions. In the beginning of this assay, it was somewhat similar to the adherence assay at the beginning. Following three rounds of washing the monolayer cells with 1x PBS, cell culture medium containing both gentamycin (100 μg/ml, Sigma) and penicillin G (5 μg/ml, Sigma) was added. The plates were then incubated for 1 h at 37°C with 5% CO_2 to kill extra-cellular and surface-adherent bacteria. Levels of association were expressed as the total number of CFU recovered per well.

THP-1 human monocytic cell line (ATCC: TIB-202, Rockville, MD, USA) was applied for intracellular survival assays, which can be kept in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, penicillin G (5 μg/ml;) and gentamicin

(100 μg/ml). It was in much similarity to that of the above invasion assays except that the initial treatment of gentamycin plus penicillin was extended to 7 h.

Assays for phagocytosis and THP-1 cell damage. Raw 264.7 murine macrophage cells and THP-1 cells were separately subjected to phagocytosis assay and cell damage test. The two kinds of experiments were conducted identically, as Segura *et al.*[30] described years ago. In short, cell cultures per well were added with 1 ml of 10^6 bacterial suspension to gain a ratio of ca. 10 bacteria per macrophage. After 1 h of incubation at 37°C, cell mono-layers were washed three times with warm PBS and re-incubated for 1 more hour with medium containing penicillin G (5 ug/ml; Sigma) and gentamicin (100 ug/ml; Sigma) to kill extra-cellular bacteria. Of note, it has already been demonstrated that these antibiotics do not penetrate eukaryotic cells under these conditions. Supernatant controls were taken in each trial to confirm that antibiotics effectively killed extra-cellular bacteria. Following the antibiotic treatment, cell mono-layers were washed three times, and dissolved in 1 ml of sterile distilled water to lyse the macrophages. After vigorous vortex, viable intracellular bacteria were determined by quantitative plating of serial dilutions of the lysates on THB agar plates.

Assays for cytokine release in THP-1 cells stimulated by *S. suis*. For evaluating the release of cytokine, THP-1 cells grown for ~48 h in flasks were collected, washed and diluted at the level of 10^5 cells/ ml, and 1 ml of this suspension was distributed in 24-well plates and incubated to confluence. At confluence, medium was removed and the designated heat-killed *S. suis* strains (1 ml) was added at appropriate dilutions. Cells incubated in medium alone served as the negative control for spontaneous cytokine release. Cytokine induction plates were maintained at 37°C, 5% CO_2 in a humid atmosphere. The ELISA method was utilized to determine the cytokine levels of culture supernatants sampled from individual wells at different time intervals.

Bioinformatics, structural and statistical analyses. The amino acid sequences of NeuB protein of different origins were collected from *E. coli*, *N. meningitidis*, and *S. suis*, respectively. The multiple alignments were conducted using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html), and the resultant output was processed by program ESPript 2.2 (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi), generating the final BLAST photography[42,48,49]. The protein sequence of the *S. suis* NeuB homologue was submitted to CPHmodels 3.0 Server (http://www.cbs.dtu.dk/services/CPHmodels), generating a PDB file of the modeled structure, which searches for a reasonable template of known structure. The tertiary structure of the modeled *S. suis* NeuB was visualized using Swiss_PDBViewer 4.0.1 software from the Swiss Institute of Bioinformatics (http://spdbv.vital-it.ch/), and was superimposed on the *N. meningitidis* NeuB structure.

All the data were expressed as means± standard deviations (SD). Unless specified, data were analyzed by two-tailed, unpaired *t* test, and all assays were repeated at least three times. A P value of <0.05 was considered as the threshold for significance.

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Author contributions

C.W., Y.F., and M.C. conceived and designed this project and experiments. J.S., Y.F. and M.C. performed the experiments and contributed to the development of the figures and tables. Y.F., M.C., J.S., H.Z., J.Z., X.Z., M.G., F.Z., X.P., F.H. and J.T. analyzed the data. Y.F., C.W. and M.C. wrote this manuscript.

Additional information

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