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Virulence traits of serogroup C meningococcus and isogenic *cssA* mutant, defective in surface-exposed sialic acid, in a murine model of meningitis

Roberta Colicchio[a], Chiara Pagliuca[a], Susanna Ricci[b], Elena Scaglione[a], Denis Grandgirard[c], Ilias Masouris[d], Fabrizio Farina[e], Caterina Pagliarulo[f], Giuseppe Mantova[a], Laura Paragliola[g], Stephen L. Leib[c], Uwe Koedel[d], Gianni Pozzi[b], Pietro Alifano[h], Paola Salvatore[a,g,i,*].

[a]Department of Molecular Medicine and Medical Biotechnology, Federico II University, Naples, Italy; [b]Laboratory of Molecular Microbiology and Biotechnology (LAMMB), Department of Medical Biotechnologies, University of Siena, Siena, Italy; [c]Institute for Infectious Diseases, University of Bern, Bern, Switzerland; [d]Department of Neurology, Ludwig-Maximilians University of Munich, München, Germany; [e]Department of Law, Economics, Management and Quantitative Methods (DEMM), University of Sannio, Benevento, Italy; [f]Department of Science and Technology, Sannio University, Benevento, Italy; [g]Department of Integrated Activity of Laboratory Medicine and Transfusion, Complex Operative Unit of Clinical Microbiology, University Hospital Federico II, Naples, Italy; [h]Department of Biological and Environmental Sciences and Technologies, University of Salento, Lecce, Italy; [i]CEINGE, Biotecnologie Avanzate s.c.ar.l., Naples, Italy.

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Address correspondence to Paola Salvatore: psalvato@unina.it

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ABSTRACT

In serogroup C *Neisseria meningitidis*, the *cssA* (*siaA*) gene codes for an UDP-*N*-acetylglucosamine 2-epimerase that catalyzes the conversion of UDP-*N*-acetyl-α-D-glucosamine into *N*-acetyl-D-mannosamine and UDP in the first step in sialic acid biosynthesis. This enzyme is required for the biosynthesis of the (α2→9)-linked polysialic acid capsule and for lipooligosaccharide (LOS) sialylation. In this study, we have used a reference serogroup C meningococcal strain and an isogenic *cssA* knockout mutant to investigate the pathogenetic role of surface-exposed sialic acids in a model of meningitis based on intracisternal inoculation of BALB/c mice. Results confirmed the key role of surface-exposed sialic acids in meningococcal pathogenesis. The 50% lethal dose (LD_{50}) of the wild type strain 93/4286 was about four orders of magnitude lower than that of the *cssA* mutant. Compared to the wild type strain, the ability of this mutant to replicate in brain and spread systemically was severely impaired. Evaluation of brain damage evidenced a significant reduction in cerebral hemorrhages in mice infected with the mutant in comparison with those challenged with the wild type strain. Histological analysis showed the typical features of bacterial meningitis, including inflammatory cells in the subarachnoid, perivascular and ventricular spaces especially in animals infected with the wild type. Noticeably, 80% of mice infected with the wild type strain presented with massive bacterial localization and accompanying inflammatory infiltrate in the *corpus callosum*, indicating high tropism of meningococci exposing sialic acids toward this brain structure and a specific involvement of the *corpus callosum* in the mouse model of meningococcal meningitis.

INTRODUCTION

Neisseria meningitidis is a leading cause of sepsis and meningitis worldwide in humans. Invasive disease is preceded by asymptomatic nasopharyngeal colonization occurring in up to 18% of the normal population. In some individuals this common transitory colonizer is able to breach the mucosal barrier, get into bloodstream and multiply uncontrollably, and finally cross the blood-brain barrier (BBB) to cause meningitis. Both host and bacterial factors seem to be involved in this switch from harmless transitory colonization to devastating disease (1).

N. meningitidis infects only humans because of the high specificity of both meningococcal surface structures and iron uptake systems for human receptors and transport proteins (2-4). The lack of valuable animal models of disease due to the narrow host range, along with the meningococcal high degree of genetic (phase and antigenic) variation of surface structures, have greatly hindered progress in understanding the pathogenesis of meningococcal disease and developing effective vaccines. Much of our knowledge about cellular and molecular biology of this human pathogen and its virulence determinants including capsular polysaccharide, lipooligosaccharide, and a number of surface-adhesive and secreted proteins comes from cell and organ culture systems or animal models that, however, fail to reproduce the complexity of the infectious cycle in the human host (5).

Among the virulence factors described so far, surface-exposed sialic acids occupy a prominent position (6,7). Thirteen *N. meningitidis* serogroups have been described on the basis of serologic differences of the capsular polysaccharides; of these, five (A, B, C, Y and W-135) cause the majority of invasive disease. According to recent WHO data, meningococcal serogroup C is still one of the most widespread serogroups in the world (Invasive Meningococcal Disease - Serogroup distribution, WHO 2018, https://www.who.int/emergencies/diseases/meningitis/en/). Recently, a new meningococcal meningitis clone of serogroup C is expanding in Sub-Saharan Africa, associated with a huge risk of a major epidemic in the next two years (WHO 2018, https://www.who.int/emergencies/diseases/meningitis/en/). In addition, since January 2015, in

74 Tuscany, Italy, there was an unexpected increase of invasive meningococcal disease, (a total of 43
75 cases, of whom 10 were fatal), due to infection with serogroup C *N. meningitidis*. Thirty-five out of
76 the samples analysed in this study were confirmed as C:P1.5–1,10–8:F3–6:ST-11 [clonal complex
77 (cc) 11] (8).

78 Among the serogroups responsible for epidemics four of them (B, C, Y and W-135) carry sialic
79 acids in their capsular polysaccharide (5). Sialic acid is also found as a modification (in place of the
80 terminal galactose residue) of the meningococcal LOS in serogroups with a sialic acid-containing
81 capsule (9). The large abundance of surface-exposed sialic acids is associated with virulence and
82 serum resistance to both phagocytosis and complement-mediated killing via alternative pathway
83 activation (10-16) resulting in enhanced survival in the bloodstream and central nervous system
84 (CNS) (17). There is also evidence that the meningococcal polysialic acid capsule is important for
85 bacterial survival within human cells (18), mediates the interaction of bacteria with host cell
86 microtubules during cell infection (19), and protects the bacteria against cationic antimicrobial
87 peptides (CAMP) including human cathelicidin LL-37 (18, 20). On the other hand, expression of
88 the polysialic acid capsule hinders colonization and invasion of the nasopharyngeal barrier by
89 masking adhesins/invasins (21-23). For these reasons, capsular polysaccharide expression is
90 subjected to frequent phase variation via slipped-strand mispairing affecting *cssD* (*siaD*) or *cssA*
91 (*siaA*) (21, 24) or reversible insertion of IS*1301* mobile elements in *cssA* (22) and is tightly
92 regulated at the transcriptional level (25). Loss or down-regulation of polysialic acid capsule
93 expression facilitates meningococcal attachment (18, 22, 23, 26) and biofilm formation, and
94 correlates with the nasopharyngeal carriage in humans (6).

95 In the past, our research group has developed a model of meningococcal meningitis (MM) based
96 on intracisternal (i.cist.) infection of adult mice with mouse-passaged bacteria (27). Survival and
97 clinical parameters of infected mice, and microbiological and histological analyses of the brain
98 demonstrated the establishment of meningitis with features comparable to those of the disease in
99 humans. Meningococci were also found in the blood, spleen, and liver of infected mice, and

bacterial loads in different organs were dependent on the infectious dose. The model was used to assess the virulence of a mutant strain deficient in the L-glutamate transporter GltT (27). The aim of the present study was to evaluate the role of surface-exposed sialic acids in the establishment of meningitis and meningoencephalitis in mice when the bacteria are directly injected i.cist using the MM model in mouse. To this purpose, we have used the reference serogroup C meningococcal strain 93/4286 and an isogenic *cssA* knockout mutant defective in UDP-*N*-acetylglucosamine 2-epimerase that catalyzes the first step of sialic acid (*N*-acetylneuraminic acid) biosynthesis, *i.e.*, the conversion of UDP-*N*-acetyl-α-D-glucosamine into *N*-acetyl-D-mannosamine and UDP (28). The 50% lethal dose (LD_{50}) of these strains were determined as well as their abilities to replicate in the brain and other organs. To investigate the infectious dynamics and histopathological correlates of the disease in the MM mouse model, histological evaluation, cerebral bleeding analysis and localization of bacteria in brain structures were carried out.

RESULTS

Construction of a serogroup C *cssA*-defective isogenic mutant. The isogenic mutant 93/4286Ω*cssA* of the serogroup C reference strain 93/4286 was obtained by insertional inactivation of the *cssA* gene (NMC0054 or NMC_RS00310), coding for the UDP-*N*-acetylglucosamine 2-epimerase (EC 3.2.1.183). In the genome of serogroup C strains, *cssA* is the first gene of the Region A capsule synthesis locus, which comprises the conserved *cssABC* (formally denominated *siaABC*) genes for CMP-*N*-acetylneuraminic acid biosynthesis followed by serogroup C-specific loci, *csc* (*siaD*$_C$) coding for α2→9 polysialyltransferase, and *cssE* (*oat*$_C$) encoding the *O*-acetyltransferase (29) (Fig. 1A). Southern blot analysis confirmed disruption of the *ccsA* gene. By using an *cssA*-specific probe, two *Nde*I DNA fragments of the expected sizes (3,180 bp and 2,183 bp) were detected in the 93/4286Ω*cssA* strain, compared to a single 791 bp *Nde*I fragment observed in the wild type strain 93/4286 (Fig. 1 B). The absence of capsular polysaccharide expression in the mutant was confirmed by latex slide agglutination test using antibodies against serogroup C meningococci capsular polysaccharide (Supplemental Fig. S1).

Characterization of 93/4286Ω*cssA* mutant under *in vitro* conditions.

In order to exclude any differences during bacterial replication, the growth rate of wild-type strain 93/4286 and its derivative *cssA* mutant was preliminarily analyzed in GC broth at 37°C. The *cssA* mutant exhibited growth curves comparable to those of the wild-type strain with a growth rate (μ= 0.97±0.08) comparable to that of the reference strain (μ= 0.85±0.06) without any statistically significant difference (Supplemental Fig. S2 and Supplemental Table S1). In GC medium, the *in vitro* competition index of *cssA* defective strain was also determined. The growth rate of mutant during the logarithmic phase of growth compared to that of the wild-type strain provided a relative fitness of the mutant of 108% compared to the wild type strain (Table 1). Moreover, the *cssA* mutant had a growth curves and colony morphology similar to those of the wild-type strain even in DMEM medium (Supplemental Fig. S3 and data not shown). In cell culture medium, the growth

rate of *cssA* defective mutant (μ= 0.25±0.004) was comparable to that of wild type strain (μ=0.30±0.04) (Supplemental Table S2).

In addition, the *cssA* mutant, grown in GC broth, exhibited a slight up-regulation in the expression of virulence-associated surface adhesins, such as pilin (*pilE*, 1.64±0.63 fold change) and non-fimbrial adhesins such as opacity protein (*opa*, 1.66±0.65 fold change), neisserial heparin–binding antigen (*nhbA*, 2.08±0.38 fold change), neisserial adhesin A (*nadA* 2.07±0.36 fold change), adhesin/invasin (*hrpA*, 1.98±0.38 fold change), and factor H binding protein (*fHbp*, 2.08±0.23 fold change) (Supplemental Fig. S4). The difference in the expression of all analyzed genes was not, however, statistically significant.

Survival of mice infected with the 93/4286Ω*cssA* mutant is significantly increased. The virulence of the *cssA*-defective strain was assessed in the MM model by analyzing animal survival at different bacterial doses. In order to determine the dose lethal for 50% of animals (LD_{50}), three groups of mice were infected by i.cist. injection of 10^4, 10^5, and 10^6 cfu of the wild type strain 93/4286 or 10^7, 10^8, and 10^9 cfu of the mutant strain 93/4286Ω*cssA*. Preliminary data showed higher survival rates in animals infected with the *cssA*-defective mutant with the same doses used for the wild type strain (data not shown). In accordance with previous results (25), mouse death, weight loss and temperature drop generally occurred within the first 72 h after meningococcal inoculation. Results with the wild type strain 93/4286 indicated that 50% and 16.6% of rodents survived meningococcal challenge with 10^4 and 10^5 cfu, respectively, while all mice died at the dose of 10^6 cfu (Fig. 2A). A significant difference was observed between the three groups (log rank test, $P < 0.05$). In contrast, at the lowest dose of 10^7 cfu, there was 83.3% survival in the group infected with the mutant strain 93/4286Ω*cssA*, while 50% survival was recorded in mice inoculated with 10^8 cfu (Fig. 2B), indicating a 10,000 folds increased LD_{50} of the *cssA*-defective mutant.

The 93/4286Ω*cssA* mutant is severely impaired at replicating in the mouse brain. To determine the number of meningococci in the brain at different stages of disease, animals were injected i.cist. with $5x10^5$ cfu of 93/4286 or 93/4286Ω*cssA* strains and sacrificed at different time points after challenge (Fig. 3A). A rapid increase in cfu counts was observed for wild type bacteria that reached the highest numbers 24 h after inoculation (8.519 log cfu±0.072). In contrast, bacterial loads in the brain of mice challenged with the *cssA*-defective mutant progressively dropped over time reaching 2.026 log cfu±1.774 72 h post infection (Fig. 3A). Bacterial clearance from the infection site occurred in 33.3% of subjects challenged with the mutant, whereas infection was never eradicated from the brain of mice that had received the wild type strain.

The *cssA*-defective mutant is cleared systemically from mice with MM. To evaluate clearance of bacteria from infected mice, two groups of animals were inoculated with $5x10^5$ cfu of either 93/4286 or 93/4286Ω*cssA* strains, and bacterial viable counts in the spleen and liver were determined (Fig. 3B). Systemic meningococcal infection caused by the *cssA*-defective mutant was entirely cleared within 48 h from i.cist. challenge, whereas none of the animals inoculated with the wild type had eliminated bacteria from spleen and liver. Two days after inoculation, mean cfu counts of the wild type strain in the spleen and liver were still 3.212 log cfu±3.354 and 6.949 log cfu±1.37, respectively. Differences in bacterial loads in the liver between the two animal groups were statistically significant (with a $P<0.001$).

Serogroup C wild type meningococci induced severe MM in mice with preferential localization in the *corpus callosum*. To compare the disease induced by the wild type 93/4286 and *cssA*-defective mutant, histological analysis and bacterial immunostaining were performed on brain slices from infected mice 48 hours after infection.

MM was considerably more severe in animals infected with the wild type (Fig. 4A) compared to those challenged with the *cssA*-defective strain (Fig. 4B). Histological analysis showed the typical

features of bacterial meningitis, including the presence of inflammatory cells in the subarachnoid (Fig. 4C, black arrowheads), perivascular and ventricular spaces (Fig. 4D, white arrowheads). Vasculitis (Fig. 4C, white arrowheads) and hemorrhages (Fig. 4A, black arrows) were observed mainly in animals infected with the wild type strain. Interestingly, inflammatory infiltrates were detected in the *corpus callosum* (Fig. 4E). Indeed, 80% of mice infected with the wild type presented with severe inflammation in the *corpus callosum* (Fig. 4E, white arrowheads). In contrast, except for one mouse, no massive evident inflammatory infiltrates, but only few immune cells, could be observed in the *corpus callosum* of animals infected with the mutant strain (Fig. 4F, white arrowheads). The presence and localization of bacteria was further investigated by immunofluorescence. In animals infected with the wild type 93/4286 strain, immunoreactivity with a meningococcal antiserum showed was mostly detected in the *corpus callosum* (Fig. 5A-B), in association with neutrophils in the ventricles (Fig. 5C) or on the meninges (data not shown). A positive signal associated with the cells lining the ventricle or possibly cells from the choroid plexus was also detected (data not shown). In contrast, immunostaining of meningococci revealed no signal in the *corpus callosum* of animals infected with the *cssA*-defective mutant (Fig. 5 D-E). A weak immunoreaction was detected in association with cells in the ventricles (Fig. 5F) or on the meninges (data not shown).

Mice infected with the *cssA*-defective mutant showed reduced intracerebral hemorrhages. In a previous study, cerebral bleeding was identified as a consistent readout in the brain of mice with MM (30). To perform a quantitative analysis of brain bleeding, the number and area of cerebral bleedings were determined in mice infected by the wild type or the *cssA*-defective strains. In accordance with histological data, results showed a significant reduction both in macroscopical assessment of cerebral haemorrhages (Fig. 6A), in the number of bleeding spots (Fig. 6B; P=0.01) and in the haemorragic area (Fig. 6C; P=0.048) in mice challenged with *cssA*-defective bacteria.

DISCUSSION

Sialic acids are a family of nine-carbon carboxylated sugars, which include more than 50 different members classified based on various substituents on carbon 4, 5, 7, 8, and 9. The substituent on carbon 5 defines the four most common types of sialic acids: neuraminic acid (Neu), N-acetylneuraminic acid (Neu5Ac), N-glycolylneuraminic acid (Neu5Gc), and 2-keto-3-deoxy-nonulosonic acid (Kdn). They can be found as terminal sugars of glycoconjugates such as glycoproteins and glycolipids on cell surface of vertebrates and higher invertebrates (31, 32). By modulating contact-dependent mechanisms, sialic acids and their metabolism play key roles in many physiological and pathological processes, including nervous system embryogenesis, regulation of immune system, cancer metastasis, bacterial and viral infection (31, 33-37). Sialic acids are also important constituents of LOS and capsular polysaccharides of some bacterial pathogens (38, 39); moreover, other bacterial pathogens not producing sialic acid, like some viruses (40), are equipped with sialidases or neuraminidases, which have been shown to be key virulence factors (41-44).

The crucial importance of sialic acids in the host-pathogen interplay is also well exemplified by our knowledge on the function and genetic regulation of polysialic acid capsule and LOS decoration with terminal sialic acid (Neu5Ac) in *N. meningitidis*. Most of the attention has been focused on the role of surface-exposed sialic acids in mediating resistance to both phagocytosis and complement-mediated killing via alternative pathway activation (10-16) resulting in enhanced meningococcal survival in the intracellular environment (18-20), in the bloodstream, and in the CNS (17). In the present study, we first aimed at validating the MM model by using a reference serogroup C strain and its attenuated isogenic *cssA* knockout mutant unable to produce sialic acids. Then, comparison of the virulence of the two strains was also instrumental to further explore the pathogenesis of MM and subsequent cerebral damage by analyzing possible interaction between meningococcal surface-exposed sialic acids and brain structures.

In this study, the inbred BALB/c mouse strain was used instead of the outbred CD-1 strain that was originally employed to establish the MM model (27). Outbred mice present with larger genetic variability that may be more suitable to uncover universal effects in a more diverse cohort and may provide results more applicable to the human population (45, 46). However, such variability requires larger sample sizes to reach sufficient statistical power and may hamper standardization procedures, and targeted studies. In our case, the LD_{50} of strain 93/4286 (without passage in mice) in inbred BALB/c mice was 10^4 cfu, while the LD_{50} in outbred CD-1 animals was approximately 10^7 cfu of mouse-passages bacteria (27). To this regard, it is noteworthy that BALB/c mice carry the s (susceptibility) mutation in the solute carrier family 11a member 1-encoding gene (*Slc11a1*), which truncates the encoded protein (also known as natural resistance-associated macrophage protein 1, Nramp1) and increases susceptibility to infection with *Mycobacteria* spp. and *Salmonella* spp. (47-51).

The key role of surface-exposed sialic acids in meningococcal pathogenesis was confirmed in the present experimental MM model. The LD_{50} of the wild type strain 93/4286 was about four orders of magnitude lower than that of the 93/4286Ω*cssA* mutant (Fig. 2). Compared to the wild type strain, the ability of the mutant to replicate in the brain (Fig. 3A) and spread systemically was severely impaired (Fig. 3B). Histological analysis and bacterial immunostaining on brain slices confirmed higher disease severity with more pronounced inflammation, vasculitis and hemorrhages in mice infected with the wild type strain compared to those challenged with the *cssA*-defective mutant (Fig. 4 and Fig. 6). The histopathological finding is reminiscent of cerebral infarction that in humans represents a complication in about 25% of patients suffering from bacterial meningitis and in 9% of MM cases (52). Interestingly, 80% of mice infected with the wild type strain 93/4286 presented with severe inflammation in the *corpus callosum* (Fig. 4), and most of the immuno-positive signal was localized in this brain structure by immunofluorescence with a meningococcal antiserum (Fig. 5). As expected, meningococci were also detected on the meninges, in the ventricles, and in the choroid plexus. Massive presence of bacteria in the vessels as well as in the epithelium of the

choroid plexus and ventricular system is a very common finding in histopathological examination of patients with MM (53, 54). Indeed, the choroid plexus is considered as an important gateway for meningococcal traversal from the bloodstream into the CNS during meningitis in humans (55). It is very likely that the bacteria utilize this highly vascularized site to spread systemically from the CNS in the i.c. mouse model of MM, by using a reverse route. In contrast, the remarkable localization of meningococci in the *corpus callosum* is unexpected, suggesting a certain tropism of *N. meningitidis* for this brain structure. On a theoretical point of view, in order to accumulate within the *corpus callosum*, in the i.c. mouse model of MM the bacteria have to leave the CSF space (since the CSF is generated within the *plexus choroideus* and flows towards the subarachnoid space), survive in the bloodstream, and re-enter into the brain. Our data seem to suggest the *corpus callosum* as a major site of bacterial re-entry in the i.c. mouse model. This could be due to a high concentration of adhesion molecules relevant to meningococcal-host cell interactions at the level of the cerebral vessels or other structures in the *corpus callosum*.

Noteworthy, there is evidence in a murine model that heparan sulfate receptors (HSPGs), which are targeted by meningococcal Opa, Opc and NhhA proteins (5) and mediate the interaction with both epithelial and endothelial cells, are highly expressed in the *corpus callosum* (56). In addition, it was also reported that the carcinoembryonic antigen-related cell-adhesion molecule-1 (CEACAM-1), which serves as a receptor for several meningococcal Opa adhesins/invasins (5) are highly expressed by oligodedrocytes, which are abundant in the *corpus callosum* (57). Furthermore, the CEACAM-1 pathway activates matrix metalloproteinases that may be involved in blood–brain-barrier breakdown (58). Noteworthy, oligodedrocytes specifically express the myelin-associated glycoprotein (MAG), which is a member of the Siglec family of proteins (sialic acid-binding, immunoglobulin-like lectins) capable of binding sialic acid (59). Thus, it is possible, although speculative, that these molecular interactions could recruit the wild type meningococci and guide they re-entry through the *corpus callosum*. In contrast, the absence of the *cssA*-defective mutant in this brain structure might be due to both/either its inability to survive in the bloodstream as

demonstrated by its complete clearance after 48 h post infection in the peripheral organs (Fig. 3B), and/or to the absence of surface-exposed sialic acid.

Although *corpus callosum* involvement as a complication of MM or invasive meningococcal disease is reported to be a rare occurrence, a case of involvement of the *corpus callosum* with cerebral ischemia and consequent callosal disconnection syndrome has been recently documented by magnetic resonance imaging and diffusion tensor tractography (60). More recently, a case of a reversible splenial lesion of the *corpus callosum* associated with MM has also been reported (61). Whether the involvement of this brain structure in meningococcal meningitis/meningoencephalitis as revealed by advanced imaging technologies may have actually been underestimated in the past, is not clear yet. In fact, the histological evidence of the localization of meningococci in the *corpus callosum* of patients who died of meningococcal disease does not yet exist. This limits our finding to the analyzed meningococcal serogroup and strain, and to the i.c. mouse model of MM used in this study.

The results of our study are consistent with the data reported by Vogel *et al.* (7) with a bacteraemia model in infant rats with serogroup B *N. meningitidis* strain B1940 and a set of isogenic mutants defective in either capsule synthesis or LOS sialylation. Infection of infant rats with the wild type strain caused severe bacteremia, while an isogenic mutant strain defective in capsule synthesis (but expressing a sialylated LOS) caused bacteremia only when a 10^6 cfu higher bacterial dose was used. In addition, when infant rats were infected with encapsulated meningococci that were unable to sialylate the LOS, bacteremia could never be induced, even with an infective dose as high as 10^8 cfu, suggesting that both forms of sialic acid on the bacterial cell surface are indispensable for systemic meningococcal survival in the infant rat model (7). Our study further expands these data to CNS infection dynamics, having, however, in mind all the limitations of an i.c. mouse model that exploits a non-natural infection route. Histological analysis and bacterial immunostaining indicates surface-exposed sialic acid as a main determinant for meningococcal intracellular growth/survival as reported before (18, 19) and also a possible

mediator in the interaction between meningococci and neuronal cells in the pathogenesis of invasive meningococcal disease. Noteworthy, sialic acid-dependent interactions play a major role in metastatic invasion of the *corpus callosum* by tumor cells. In humans, the frequency of polysialic acid-positive cells and polysialyltransferase expression were higher in diffuse and recurrent astrocytoma (associated with the invasion of the *corpus callosum*) than in astrocytoma with lower spreading potential (62). This study suggests that tumor cells expressing polysialic acid on neural cell adhesion molecules (NCAMs) may interact with adhesive receptors in the *corpus callosum*, thus allowing tumor cell migration and localization in this brain structure (62). Similar molecular interactions may explain the massive localization of wild type meningococci in the *corpus callosum* in our MM model, proposing a new role of microbial surface-exposed sialic acids in the interplay between *N. meningitidis* and the host in the pathogenesis of meningococcal disease that, however, should be further explored.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The meningococcal strains used in this study are the serogroup C strain 93/4286 and the sialic acid deficient isogenic mutant 93/4286Ω*cssA*. The 93/4286 strain belonging to the ET-37 hypervirulent lineage (cc ST-11) was kindly provided by Novartis Vaccine and Diagnostics, Siena, Italy. Meningococci were cultured on gonococcus (GC) (Oxoid S.p.A., Milan, Italy) agar/broth supplemented with 1% (vol/vol) Polyvitox (Oxoid) at 37°C with 5% CO_2. When needed, erythromycin (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) was added to a final concentration of 7 µg ml^{-1}. Meningococci were also cultured in Dulbecco's modified Eagle medium (DMEM) (Microgem, Naples, Italy) with 10% fetal bovine serum heat inactivated (Microgem) and 2 mM L-glutamine (Microgem). To evaluate the fitness of each strain, at every stage of growth, serial dilutions were plated on GC agar in the presence or absence of erythromycin and incubated at 37°C with 5% CO_2 for 24 h. After growth, viable cell counts were determined by the CFU method. The growth rate µ (h^{-1}) of the *cssA* mutant and the wild-type strain was calculated as described by Barlow and coworkers (63), and the number of generation (G) and *t* per generation were calculated as described by Gillespie and coworkers (64).

All experiments were performed in triplicate with three independent cultures, the results obtained were analyzed and graphically reported by using GraphPad Prism (v.4) software, and statistical significance was examined by the Student's *t*-test. Pairwise competition experiments were used to estimate the *in vitro* fitness of *cssA* defective mutant relative to that of the wild type strain. Equal numbers of CFU of the isogenic mutant and wild type strains were mixed together (1:1), and the bacteria were allowed to grow together competitively in antibiotic-free GC broth at 37°C. Experiments were conducted as previously described (65).

Inocula for mouse challenge were prepared by cultivating bacteria in GC broth until mid-logarithmic phase. Viable cell counts were determined, and bacteria were frozen at -80°C with 10% glycerol until use. *Escherichia coli* strain DH5α was used in cloning procedures. This strain was

grown in Luria-Bertani (LB) (Oxoid) medium. To allow plasmid selection, LB medium was supplemented with ampicillin (50µg ml^{-1}) (Sigma-Aldrich, Merck KGaA).

DNA procedures, plasmids and transformation of *N. meningitidis*. High-molecular-weight genomic DNA from *N. meningitidis* strains was prepared as previously reported (66). DNA fragments were isolated by using acrylamide slab gels and recovered by electroelution as described before (67). Oligonucleotide synthesis and DNA sequencing were performed by Ceinge-Advanced Biotechnologies, Naples, Italy. DNA sequence analysis was carried out by using the GeneJockey Sequence Processor software (Biosoft) and multiple sequence alignment tool Clustal W (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

To construct the pDEΔ*cssA* vector, a genomic fragment of *cssA* [also known as *siaA*, *synA* or *neuA* before proposal of a unified nomenclature for capsule loci (29)] (644 bp) was amplified from genomic DNA of 93/4286 strain using the primers CssAXbaF (5'-ATTGAACCTCTAGAGGTCATGATTCACGGCGACCG-3') and CssAXbaR (5'-TGGCGTTCTAGAACATCAATTGAAGGGACACCG-3'). Amplification reactions were as follows: 45 s of denaturation at 94°C, 45 s of annealing at 65°C, and 60 s of extension at 72°C for a total of 30 cycles. Reactions were carried out in a MyCycler thermal cycler (Bio-Rad, Laboratories S.r.l., Segrate, Milan, Italy). The amplicon was cloned into the *Xba*I site of *Neisseria-E. coli* shuttle plasmid pDEX (66, 68). Plasmid pDEΔ*cssA* was then used to genetically inactivate by single cross-over the *cssA* gene (NMC0054or NMC_RS00310) of strain 93/4286 coding for the UDP-*N*-acetylglucosamine 2-epimerase. Transformation experiments were performed using 0.1 to 1 µg of plasmid DNA as previously described (69). Transformants were selected on GC agar medium supplemented with erythromycin. Gene disruption was demonstrated by Southern blot hybridization using a 644-bp-long *cssA*-specific ^{32}P-labeled probe. Southern blot hybridizations were carried out according to standard protocols (67). ^{32}P-labeling of DNA fragments was performed by random priming using the Klenow fragment of *E. coli* DNA polymerase I and [α-^{32}P] dGTP (3,000 Ci

mmol^{-1}) (67). To detect capsular polysaccharide expression, a rabbit polyclonal antibody-coated latex suspension against groups C and W135 *N. meningitidis* was used by latex slide agglutination test (BD Directigen™ Meningitis Combo Test, BD Italia, Milan, Italy).

Real-time RT-PCR experiments. Semi-quantitative analysis of the *pilE, fHbp, hrpA, nadA, opa54, nhbA* transcript normalized to the level of expression of the *16S* rRNA gene (65) was performed by real-time reverse transcriptase (RT)-PCR. Wild-type strain 93/4286 and *cssA* defective mutant were grown to late logarithmic phase (OD_{600}, 1.0) in GC broth. Total bacterial RNAs were then extracted by use of an RNeasy minikit (Qiagen, Venlo, the Netherlands) according to the manufacturer's instructions. Before extraction, samples were treated with 2 volumes of RNA Protect Bacteria reagent (Qiagen). DNA contamination was avoided by on-column treatment with an RNase-free DNase set (Qiagen) according to the manufacturer's instructions. This procedure was performed in triplicate for each strain. The concentration and integrity of the RNA samples were assessed by measurement of the A_{260}/A_{280} and A_{260}/A_{230} ratios and were verified using a NanoDrop Lite spectrophotometer (ThermoFisher Scientific, Waltham, Massachusetts, USA). Then, for each sample, total RNAs (2γ) were reverse transcribed into cDNA as previously described (65). For semiquantitative analysis, about 64-128 ng of each reverse transcription reaction mixture was used to run a real-time PCR on an Applied Byosistems 7500 Fast Real-Time PCR System (Applied Byosistems, Foster City, California, USA) with KiCqStart™ SYBR Green qPCR Ready Mix™, Low ROX™ (Sigma-Aldrich) and with specific primers pair as reported in Supplemental Table S3.

PCR was conducted according to the manufacturer's guidelines as following reported: initial holding and activation at 95°C for 10 min, PCR was performed for 40 cycles at 95°C for 20 s, 60°C for 30 s and 72°C for 30 s. The post-PCR melt curve was performed between temperatures of 60°C to 95°C with 1% temperature increments. Previously, standard curves were analyzed to determine the efficiency of amplification and the $2^{-\Delta\Delta CT}$ method was used for the analysis of the level (n-fold) of change. Samples were run in the real-time PCR in triplicate, and statistical significance was

examined by Mann-Whitney U test. To quantify gene expression, we expressed the data as fold change obtained using the $2^{-\Delta\Delta CT}$ method, where CT is threshold cycle.

Mice, MM model and experimental design. Eight-week-old female inbred BALB/c mice weighing 19 to 20 g were purchased from Charles River Italia (Lecco, Italy). The mice were fed with laboratory food pellets and tap water *ad libitum*, and were housed under specific pathogen free conditions. All efforts were made to minimize animal suffering and reduce the number of mice in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC). The study was approved by the Ethical Animal Care and Use Committee (Prot. number 2, 14 December 2012) and the Italian Ministry of Health (Prot. number 0000094-A-03/01/2013). For brain histology and cerebral bleeding analysis, animal experiments were performed at the Azienda Ospedaliera Universitaria Senese and authorised by the Local Ethics Committee (Comitato Etico Locale, Azienda Ospedaliera Universitaria Senese, 21.05.2012) and the Italian Ministry of Health (Document no. 131/2013, 30.05.2013).

Mice were infected by the i.cist. route as previously described (27, 30, 70). Bacteria for mouse challenge were prepared as previously reported (27), thawed, centrifuged for 15 min at 1500 x *g*, and suspended in GC broth with iron dextran (5 mg/kg; Sigma-Aldrich, Merck KGaA). Approximately 2 h before infection, animals were injected intraperitoneally (i.p.) with iron dextran (250 mg kg^{-1}). Animals were lightly anesthetized (50 mg/kg ketamine and 3 mg/kg xylazine or Zoletil 20 [30 mg/kg; VirbacSrl] and Xilor [8 mg/kg; Bio 98 Srl]), and bacteria (suspended in a total volume of 10 µl) were inoculated by hand-puncturing the *cisterna magna* of mice using a 30-gauge needle (BD Italia, Milan, Italy). Mice were monitored for possible seizures due to inoculation. Clinical signs were monitored according to a previously described coma scale (71), and mice with a score of 2 were euthanized and recorded as dead for statistical analysis .

For brain histology and cerebral bleeding analysis, brains were removed and dissected into the two hemispheres and cerebellum. One hemisphere was fixed in 4% paraformaldehyde (PFA) in

Phosphate-Buffered Saline (PBS) (w/v) for histological analysis, while the other one was frozen in dry ice for assessment of intracerebral bleeding. Samples were not collected from animals found dead or humanely sacrificed before 48 h.

Animal survival and cfu counts. Different bacterial doses ranging from 10^4 to 10^6 cfu per mouse for the 93/4286 wild type strain, and from 10^7 to 10^9 for the 93/4286Ω*cssA* isogenic mutant were used to inoculate animals (n=6/dose). Control mice were inoculated with GC broth. Every day throughout the whole experiment, animals were monitored for clinical signs, and body weight and temperature were measured as previously described (27). Survival was recorded for a week. To determine the number of meningococci in the brain over time, animals were infected with $5x10^5$ cfu/mouse and sacrificed at different time points (4, 24, 48, and 72 h) (n=3/time point) after infection. To compare the virulence of the wild type strain versus the *cssA*-defective mutant, two groups of mice (n=5/group) were infected with $5x10^5$ cfu/mouse and sacrificed 48 h after challenge for organ collection. Brain, spleen, and liver were excised and homogenized in 1 ml of GC medium. Viable cell counts were performed by plating 10-fold dilutions onto GC agar plates with (mutant) or without (wild type) erythromycin.

Brain histology. Experiments were performed with a total number of 20 mice, of which 8 were infected with the wild type strain and 8 were challenged with the *cssA*-defective mutant, and four served as control. Infection dose was $6x10^5$ cfu/mouse. Brain hemispheres from mice infected for 48 hours were prepared for cryopreservation by incubation in 18% sucrose in PBS (w/v) at 4 ºC overnight. Hemispheres were mounted in OCT compound and cut coronally using a Leica 3050S cryostat (Leica Biosystems, Wetzlar, Germany). Forty-five µm-thick coronal sections were sampled at a frequency of every 15^{th} slice. Additional 10 µm sections were obtained for immunofluorescence analysis. Histopathological evaluations were made on sections stained with cresyl violet for Nissl substance.

461

462 **Immunofluorescence.** Slices were incubated with a primary rabbit polyclonal antibody against
463 whole-cell preparation of serogroup A, B, and C *N. meningitidis* (ViroStat, cat. # 6122, Portland,
464 ME, USA) at a dilution of 1:1000. This antibody was reactive against both encapsulated and
465 unencaspulated meningococci (18, 19). Sections were washed three times with PBS and incubated
466 with the secondary antibody (goat anti-rabbit Cy3; Jackson, West Grove, PA, USA) for 45 min at
467 room temperature in the dark. Primary and secondary antibodies were diluted in Tris Buffered
468 Saline (TBS; Sigma-Aldrich, Merck KGaA) containing 0.5% bovine serum albumin. After washing,
469 slides were counterstained with DAPI for 1 min, washed and mounted with Mowiol® (Merck)
470 containing 2.5% Dabco®(Sigma-Aldrich, Merck KGaA). Pictures were obtained using a Zeiss
471 fluorescent microscope (AxioImager M1, Zeiss, West Germany) equipped with a digital camera
472 (AxioCamHRc). Overview pictures were created by combining photos obtained with a 10x
473 objective and mosaic reconstruction using the AxioVision 4.8 software (Zeiss, Oberkochen,
474 Germany).

475

476 **Analysis of cerebral bleeding.** Cerebral haemorrhages were assessed as previously described (72).
477 Briefly, brain hemispheres were cut in a frontal plane into 30 µm-thick sections, and serial sections
478 were photographed with a digital camera at 0.3 mm-intervals. For each animal, 5 comparable brain
479 sections were analyzed. The bleeding spots were counted, and the relative areas of bleeding were
480 measured by using the UTHSCSA Image Tool (Texas, USA). Cumulative bleeding areas were
481 divided by the whole slice area and computed into a total bleeding area/whole slice area x 1000.

482

483 **Statistical analysis.** Bacterial counts in different organs and time-points were represented as mean
484 ± SD of cfu numbers isolated from single mice. Differences in growth rates in *in vitro* experiments
485 and differences in bacterial loads between mice infected with the wild type strain or the mutant
486 were examined by the Student's *t*-test. Mouse survival was estimated by the Kaplan-Meier survival

analysis, and differences were compared using the log-rank test ($P<0.05$). Differences in expression of surface adhesins by RT Real time PCR and differences in cerebral bleeding were evaluated using the Mann-Whitney U test ($P<0.05$).

Table 1. Competition assay of 93/4286Ω*cssA* defective mutant strain with wild type strain.

Mutant strain	No. of generations[a]		Cost per generation[b]	$D_{0\text{-}1.0OD}$ [b,c]	Relative fitness (g_R/g_S)[b]
	g_S	g_R			
93/4286Ω*cssA*	9.93 ± 0.68	10.77 ± 0.85	-0.058 ± 0.01	0.057 ± 0.01	1.08 ± 0.02

[a] The number of generations of the mutant strain (g_R) and of the wild-type strain (g_S) was calculated as described by Gillespie and coworkers (64). The values are the means ± SD_s from four independent experiments.

[b] The values are presented as the means ± SD_s from four independent experiments.

[c] The difference in fitness ($D_{0\ -1.0OD}$) was estimated by direct competition against an equal number of CFU of the mutant - wild-type strain.

SUPPLEMENTAL MATERIAL

SUPPLEMENTAL FILE 1, PDF file, 810KB

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REFERENCE

1. van Deuren M, Brandtzaeg P, van der Meer JW. 2000. Update on meningococcal disease with emphasis on pathogenesis and clinical management. Clin Microbiol Rev 13:144-166.

2. Plant L, Jonsson AB. 2003. Contacting the host: insights and implications of pathogenic *Neisseria* cell interactions. Scand J Infect Dis 35:608-613.

3. Schryvers AB, Stojiljkovic I. 1999. Iron acquisition systems in the pathogenic *Neisseria*. Mol Microbiol 32:1117-1123.

4. Virji M, Makepeace K, Ferguson DJ, Watt SM. 1996. Carcinoembryonic antigens (CD66) on epithelial cells and neutrophils are receptors for Opa proteins of pathogenic neisseriae. Mol Microbiol 22:941-950.

5. Hill DJ, Griffiths NJ, Borodina E, Virji M. 2010. Cellular and molecular biology of *Neisseria meningitidis* colonization and invasive disease. ClinSci (Lond) 118:547-564.

6. Tzeng YL, Thomas J, Stephens DS. Regulation of capsule in *Neisseria meningitidis*. 2015. Crit Rev Microbiol 19:1-14.

7. Vogel U, Hammerschmidt S, Frosch M. 1996. Sialic acids of both the capsule and the sialylated lipooligosaccharide of *Neisseria meningitis* serogroup B are prerequisites for virulence of meningococci in the infant rat. Med Microbiol Immunol 185:81-87.

8. Stefanelli P, Miglietta A, Pezzotti P, Fazio C, Neri A, Vacca P, Voller F, D'Ancona FP, Guerra R, Iannazzo S, Pompa MG, Rezza G. 2016. Increased incidence of invasive

meningococcal disease of serogroup C / clonal complex 11, Tuscany, Italy, 2015 to 2016. Euro Surveill 21:(12).

9. Mandrell RE, Kim JJ, John CM, Gibson BW, Sugai JV, Apicella MA, Griffiss JM, Yamasaki R. 1991. Endogenous sialylation of the lipooligosaccharides of *Neisseria meningitidis*. J Bacteriol 173:2823-2832.

10. Estabrook MM, Christopher NC, Griffiss JM, Baker CJ, Mandrell RE. 1992. Degree of endogenous LOS sialylation and amount of sialic acid capsule were associated with each other and with susceptibility to killing by neutrophils for the non-2b:P1.2 strains. J Infect Dis 166:1079-1088.

11. Estabrook MM, Griffiss JM, Jarvis GA. 1997. Sialylation of *Neisseria meningitidis* lipooligosaccharide inhibits serum bactericidal activity by masking lacto-N-neotetraose. Infect Immun 65:4436-4444.

12. Hammerschmidt S, Birkholz C, Zähringer U, Robertson BD, van Putten J, Ebeling O, Frosch M. 1994. Contribution of genes from the capsule gene complex (*cps*) to lipooligosaccharide biosynthesis and serum resistance in *Neisseria meningitidis*. Mol Microbiol 11:885-896.

13. Jarvis GA. 1995. Recognition and control of neisserial infection by antibody and complement. Trends Microbiol 3:198-201.

14. John CM, Phillips NJ, Din R, Liu M, Rosenqvist E, Høiby EA, Stein DC, Jarvis GA. 2016. Lipooligosaccharide structures of invasive and carrier isolates of *Neisseria meningitidis* are correlated with pathogenicity and carriage. J Biol Chem 291:3224-3238.

15. Lewis LA, Carter M, Ram S. 2012. The relative roles of factor H binding protein, neisserial surface protein A, and lipooligosaccharide sialylation in regulation of the alternative pathway of complement on meningococci. J Immunol 188:5063–5072.

16. Ram S, Mackinnon FG, Gulati S, McQuillen DP, Vogel U, Frosch M, Elkins C, Guttormsen HK, Wetzler LM, Oppermann M, Pangburn MK, Rice PA. 1999. The contrasting mechanisms of serum resistance of *Neisseria gonorrhoeae* and group B *Neisseria meningitidis*. Mol Immunol 36:915–928.

17. Stephens DS. 2009. Biology and pathogenesis of the evolutionarily successful,obligate human bacterium *Neisseria meningitidis*. Vaccine 27 Suppl 2 :B71-7.

18. Spinosa MR, Progida C, Tala A, Cogli L, Alifano P, Bucci C. 2007. The *Neisseria meningitidis* capsule is important for intracellular survival in human cells. Infect Immun 75:3594-3603.

19. Talà A, Cogli L, De Stefano M, Cammarota M, Spinosa MR, Bucci C, Alifano P. 2014. Serogroup-specific interaction of *Neisseria meningitidis* capsular polysaccharide with host cell microtubules and effects on tubulin polymerization. Infect Immun 82(1):265-274.

20. Jones A, Geörg M, Maudsdotter L, Jonsson AB. 2009. Endotoxin, capsule, and bacterial attachment contribute to *Neisseria meningitidis* resistance to the human antimicrobial peptide LL-37. J Bacteriol 191:3861-3868.

21. Hammerschmidt S, Müller A, Sillmann H, Mühlenhoff M, Borrow R, Fox A, van Putten J, Zollinger WD, Gerardy-Schahn R, Frosch M. 1996a. Capsule phase variation in *Neisseria meningitidis* serogroup B by slipped-strand mispairing in the polysialyltransferase gene (*siaD*): correlation with bacterial invasion and the outbreak of meningococcal disease. Mol Microbiol 20:1211-1220.

22. Hammerschmidt S, Hilse R, van Putten JP, Gerardy-Schahn R, Unkmeir A, Frosch M. 1996b. Modulation of cell surface sialic acid expression in *Neisseria meningitidis* via a transposable genetic element. EMBO J 15:192-198.

23. Unkmeir A, Kämmerer U, Stade A, Hübner C, Haller S, Kolb-Mäurer A, Frosch M, Dietrich G. 2002. Lipooligosaccharide and polysaccharide capsule: virulence factors of *Neisseria meningitidis* that determine meningococcal interaction with human dendritic cells. Infect Immun 70:2454-2462.

24. Weber MV, Claus H, Maiden MC, Frosch M, Vogel U. 2006. Genetic mechanisms for loss of encapsulation in polysialyltransferase-gene-positive meningococci isolated from healthy carriers. Int J Med Microbiol 296:475-484.

25. Deghmane AE, Giorgini D, Larribe M, Alonso JM, Taha MK. 2002. Down-regulation of pili and capsule of *Neisseria meningitidis* upon contact with epithelial cells is mediated by CrgA regulatory protein. Mol Microbiol 43:1555-1564.

26. Bartley SN, Tzeng YL, Heel K, Lee CW, Mowlaboccus S, Seemann T, Lu W, Lin YH, Ryan CS, Peacock C, Stephens DS, Davies JK, Kahler CM. 2013. Attachment and invasion of *Neisseria meningitidis* to host cells is related to surface hydrophobicity, bacterial cell size and capsule. PLoS One 8(2):e55798.

27. Colicchio R, Ricci S, Lamberti F, Pagliarulo C, Pagliuca C, Braione V, Braccini T, Talà A, Montanaro D, Tripodi S, Cintorino M, Troncone G, Bucci C, Pozzi G, Bruni CB, Alifano P, Salvatore P. 2009. The meningococcal ABC-Type L-glutamate transporter GltT is necessary for the development of experimental meningitis in mice. Infect Immun 77:3578-3587.

28. Murkin AS, Chou WK, Wakarchuk WW, Tanner ME. 2004. Identification and mechanism of a bacterial hydrolyzing UDP-N-acetylglucosamine 2-epimerase. Biochemistry 43:14290-14298.

29. Harrison OB, Claus H, Jiang Y, Bennett JS, Bratcher HB, Jolley KA, Corton C, Care R, Poolman JT, Zollinger WD, Frasch CE, Stephens DS, Feavers I, Frosch M, Parkhill J, Vogel U, Quail MA, Bentley SD, Maiden MC. 2013. Description and nomenclature of *Neisseria meningitidis* capsule locus. Emerg Infect Dis 19:566-573.

30. Ricci S, Grandgirard D, Wenzel M, Braccini T, Salvatore P, Oggioni MR, Leib SL, Koedel U. 2014. Inhibition of matrix metalloproteinases attenuates brain damage in experimental meningococcal meningitis. BMC Infect Dis 14:726.

31. Chen X, Varki A. 2010. Advances in the biology and chemistry of sialic acids. ACS Chem Biol 5:163-176.

32. Varki NM, Strobert E, Dick EJ Jr, Benirschke K, Varki A. 2011. Biomedical differences between human and nonhuman hominids: potential roles for uniquely human aspects of sialic acid biology. Annu Rev Pathol 6:365-393.

33. Angata T, Varki A. 2002. Chemical diversity in the sialic acids and related alpha-keto acids: an evolutionary perspective. Chem Rev 102:439-469.

34. Chen GY, Chen X, King S, Cavassani KA, Cheng J, Zheng X, Cao H, Yu H, Qu J, Fang D, Wu W, Bai XF, Liu JQ, Woodiga SA, Chen C, Sun L, Hogaboam CM, Kunkel SL, Zheng P, Liu Y. 2011. Amelioration of sepsis by inhibiting sialidase-mediated disruption of the CD24-SiglecG interaction. Nat Biotechnol 29:428-435.

35. Li Y, Chen X. 2012. Sialic acid metabolism and sialyltransferases: natural functions and applications. Appl Microbiol Biotechnol 94:887-905.

36. Chang YC, Nizet V. 2014. The interplay between Siglecs and sialylated pathogens. Glycobiology. 24:818-825.

37. Petridis AK, El-Maarouf A, Rutishauser U. 2004. Polysialic acid regulates cell contact-dependent neuronal differentiation of progenitor cells from the subventricular zone. Dev Dyn 230:675-684.

38. Severi E, Hood DW, Thomas GH. 2007. Sialic acid utilization by bacterial pathogens. Microbiology 153:2817-2822.

39. Vimr ER, Kalivoda KA, Deszo EL, Steenbergen SM. 2004. Diversity of microbial sialic acid metabolism. Microbiol Mol Biol Rev 68:132-153.

40. Suzuki Y. 2005. Sialobiology of influenza: molecular mechanism of host range variation of influenza viruses. Biol Pharm Bull 28:399-408.

41. Amano A, Chen C, Honma K, Li C, Settem RP, Sharma A. 2014. Genetic characteristics and pathogenic mechanisms of periodontal pathogens. Adv Dent Res 26:15-22.

42. Camara M, Boulnois GJ, Andrew PW, Mitchell TJ. 1994. A neuraminidase from *Streptococcus pneumoniae* has the feature of a surface protein. Infect Immun 62:3688-3695.

43. Gualdi L, Hayre JK, Gerlini A, Bidossi A, Colomba L, Trappetti C, Pozzi G, Docquier JD, Andrew P, Ricci S, Oggioni MR. 2012. Regulation of neuraminidase expression in *Streptococcus pneumoniae*. BMC Microbiol 12:200.

44. Juge N, Tailford L, Owen CD. 2016. Sialidases from gut bacteria: a mini-review. Biochem Soc Trans 44:166-175.

45. Festing MFW. 1976. Phenotypic variability of inbred and outbred mice. Nature 263:230-232.

46. Festing MFW. 1999. Warning: the use of heterogeneous mice may seriously damage your research. Neurobiol Aging 20: 237-244.

686 47. Medina E, North RJ. 1996a. Evidence inconsistent with a role for the *Bcg* gene (Nramp1) in
687 resistance of mice to infection with virulent *Mycobacterium tuberculosis*. J Exp Med
688 183:1045-1051.
689
690 48. Medina E, North RJ. 1996b. Mice that carry the resistance allele of the *Bcg* gene (Bcgr)
691 develop a superior capacity to stabilize bacilli Calmette-Guerin (BCG) infection in their
692 lungs and spleen over a protracted period in the absence of specific immunity. Clin Exp
693 Immunol 104:44-47.
694
695 49. Medina E, North RJ. 1999. Genetically susceptible mice remain proportionally more
696 susceptible to tuberculosis after vaccination. Immunology 96:16-21.
697
698 50. Vidal SM, Malo D, Vogan K, Vogan K, Skamene E, Gros P. 1993. Natural resistance to
699 infection with intracellular parasites: isolation of a candidate for Bcg. Cell 73:469-485.
700
701 51. Vidal S, Tremblay ML, Govoni G, Gauthier S, Sebastiani G, Malo D, Skamene E, Olivier
702 M, Jothy S, Gros P. 1995. The Ity/Lsh/Bcg locus: natural resistance to infection with
703 intracellular parasites is abrogated by disruption of the Nramp1 gene. J Exp Med 182:655-
704 666.
705
706 52. Schut ES, Lucas MJ, Brouwer MC, Vergouwen MD, van der Ende A, van de Beek D. 2012.
707 Cerebral infarction in adults with bacterial meningitis. Neurocrit Care 16:421-427.
708
709 53. Guarner J, Greer PW, Whitney A, Shieh WJ, Fischer M, White EH, Carlone GM, Stephens
710 DS, Popovic T, Zaki SR. 2004. Pathogenesis and diagnosis of human meningococcal
711 disease using immunohistochemical and PCR assays. Am J Clin Pathol 122:754-764.

54. Pron B, Taha MK, Rambaud C, Fournet JC, Pattey N, Monnet JP, Musilek M, Beretti JL, Nassif X. 1997. Interaction of *Neisseria maningitidis* with the components of the blood-brain barrier correlates with an increased expression of PilC. J Infect Dis 176:1285-1292.

55. Schwerk C, Tenenbaum T, Kim KS, Schroten H. 2015. The choroid plexus-a multi-role player during infectious diseases of the CNS. Front Cell Neurosci 9:80.

56. Kaur C, Sivakumar V, Yip GW, Ling EA. 2009. Expression of syndecan-2 in the amoeboid microglial cells and its involvement in inflammation in the hypoxic developing brain. Glia. 57(3):336-49.

57. Neyazi B, Herz A, Stein KP, Gawish I, Hartmann C, Wilkens L, Erguen S, Dumitru CA, Sandalcioglu IE. 2017. Brain arteriovenous malformations: implications of CEACAM1-positive inflammatory cells and sex on hemorrhage. Neurosurg Rev. 40(1):129-134.

58. Ludewig P, Sedlacik J, GelderblomM, Bernreuther C, Korkusuz Y, Wagener C, Gerloff C, Fiehler J, Magnus T, Horst AK. 2013. Carcinoembryonic antigen-related cell adhesion molecule 1 inhibits MMP-9-mediated blood–brain-barrier breakdown in a mouse model for ischemic stroke. Circ Res 113:1013-1022

59. Huang JY, Wang YX, Gu WL, Fu SL, Li Y, Huang LD, Zhao Z, Hang Q, Zhu HQ, Lu PH. 2012. Expression and function of myelin-associated proteins and their common receptor NgR on oligodendrocyte progenitor cells. Brain Res 1437:1-15.

60. Marchi NA, Ptak R, Wetzel C, Vargas MI, Schnider A, Nicastro N. 2016. Callosal disconnection syndrome after ischemic stroke of the corpus callosum due to meningococcal meningitis: A case report. J Neurol Sci 369:119-120.

61. Hayashi Y, Yasunishi M, Hayashi M, Asano T, Kimura A, Inuzuka T. 2017. Reversible splenial lesion of the corpus callosum associated with meningococcal meningitis. J Neurol Sci 373:81-82.

62. Suzuki M, Suzuki M, Nakayama J, Suzuki A, Angata K, Chen S, Sakai K, Hagihara K, Yamaguchi Y, Fukuda M. 2005. Polysialic acid facilitates tumor invasion by glioma cells. Glycobiology 15:887-894.

63. Hall BG, Acar H, Nandipati A, Barlow M. 2014. Growth rates made easy. Mol Biol Evol. 31(1):232-8.

64. Billington OJ, McHugh TD, Gillespie SH. 1999. Physiological cost of rifampin resistance induced in vitro in Mycobacterium tuberculosis. Antimicrob Agents Chemother. 43(8):1866-9.

65. Colicchio R, Pagliuca C, Pastore G, Cicatiello AG, Pagliarulo C, Talà A, Scaglione E, Sammartino JC, Bucci C, Alifano P, Salvatore P. 2015. Fitness Cost of Rifampin Resistance in *Neisseria meningitidis*: In Vitro Study of Mechanisms Associated with rpoB H553Y Mutation. Antimicrob Agents Chemother. 59(12):7637-49.

66. Bucci C, Lavitola A, Salvatore P, Del Giudice L, Massardo DR, Bruni CB, Alifano P. 1999. Hypermutation in pathogenic bacteria: frequent phase variation in meningococci is a phenotypic trait of a specialized mutator biotype. Mol Cell 3:435-445.

67. Sambrook J, Russell, DW. 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

68. Pagliarulo C, Salvatore P, De Vitis LR, Colicchio R, Monaco C, Tredici M, Talà A, Bardaro M, Lavitola A, Bruni CB, Alifano P. 2004. Regulation and differential expression of *gdhA* encoding NADP-specific glutamate dehydrogenase in *Neisseria meningitidis* clinical isolates. Mol Microbiol 51:1757-1772.

69. Frosch M, Schultz E, Glenn-Calvo E, Meyer TF. 1990. Generation of capsule-deficient *Neisseria meningitidis* strains by homologous recombination. Mol Microbiol 4:1215-1218.

70. Koedel U, Paul R, Winkler F, Kastenbauer S, Huang PL, Pfister HW. 2001. Lack of endothelial nitric oxide synthase aggravates murine pneumococcal meningitis. J Neuropathol Exp Neurol 60:1041-1050.

71. Liechti FD, Grandgirard D, Leppert D, Leib SL. 2014. Matrix metalloproteinase inhibition lowers mortality and brain injury in experimental pneumococcal meningitis. Infect Immun 82:1710-1718.

72. Koedel U, Frankenberg T, Kirschnek S, Obermaier B, Häcker H, Paul R, Häcker G. 2009. Apoptosis is essential for neutrophil functional shutdown and determines tissue damage in experimental pneumococcal meningitis. PLoS Pathog 5:e1000461.

787 **Figure Legends**

788 **FIG 1.** Knock out of the *cssA* gene in the 93/4286 strain. Experimental design for *cssA* disruption
789 by single crossing-over (A). The genetic map of the *cps* locus Region A (capsule synthesis) of *N.*
790 *meningitidis* serogroup C was constructed on the basis of the available nucleotide sequences of
791 FAM18 (ET-37) in the NCBI data bank (https://www.ncbi.nlm.nih.gov/nuccore/NC_008767) with
792 arrows depicting gene orientation. The genetic determinants of plasmid pDEΔ*cssA* are: i) a DNA
793 fragment containing a DUS, required for efficient DNA uptake during transformation; ii) *ermC*, the
794 erythromycin resistance gene used as a selective marker for transformation; iii) Δ*cssA*, a 644 bp
795 *Xba*I DNA fragment spanning the central part of *cssA* and iv) black box, that indicates the plasmid
796 polylinker region. The physical map of the pDEΔ*cssA* plasmid is also indicated. Southern blot
797 analysis demonstrating inactivation of *cssA* (B). Chromosomal DNA was extracted from the
798 parental strains 93/4286 (lane 1) and an isogenic mutant, 93/4286Ω*cssA* (lane 2), obtained by
799 transformation with pDEΔ*cssA* and selection with erythomycin. Chromosomal DNA was analyzed
800 by Southern blot using a *cssA*-specific probe. Bars on the right indicate *cssA*-specific fragments
801 whose sizes were deduced on the basis of the relative migration pattern of DNA ladders (bars on the
802 left).
803
804 **FIG 2.** Survival of mice infected with wild type or *cssA*-defective *N. meningitidis* strains. Three
805 groups of BALB/c mice (n= 6/dose) were infected i.cist. with 10^4, 10^5, and 10^6 cfu per mouse of the
806 wild type strain 93/4286 (A) and with 10^7, 10^8, and 10^9 cfu per mouse of the *cssA*-defective mutant
807 (B). Mice were monitored for a week, and survival was recorded. Results are expressed as percent
808 survival at different doses over time, the log rank p value was < 0.05 for mice infected with the wild
809 type strain.
810
811 **FIG 3.** Bacterial loads over time in mice inoculated with the 93/4286 or 93/4286Ω*cssA* strains.
812 Time course of bacterial loads in the brain following i.cist. infection (A). Two groups of BALB/c

mice (n=20/group) were infected by the i.cist. route with $5x10^5$ cfu of either the wild type strain 93/4286 or the *cssA*-defective mutant. Animals were sacrificed 4, 24, 48, and 72 h after infection (3/time point). Brains were collected, homogenized in GC medium, and viable counts were determined. Results are expressed as mean ±SD log of cfu numbers per organ at different time points after inoculation. Asterisks indicate statistical significance (**, $P<0.01$). Bacterial loads over time in spleen and liver (B). Two groups of BALB/c mice (n=5/group) were infected i.cist. with $2x10^6$ cfu of either the wild type strain 93/4286 or the *cssA*-defective mutant. Animals were sacrificed 48 h after infection. Spleens and livers were collected, homogenized, and viable counts were determined. Results are expressed as log cfu numbers per organ. Horizontal bars indicate mean logs of bacterial titers. Each symbol represents a single animal. Asterisks indicate statistical significance (***, $P<0.001$).

FIG 4. Cresyl violet stained sections of brains from animals mice infected with wild type or *cssA*-defective *N. meningitidis* strains. BALB/c mice were challenged by the i.cist. route with either the wild type 93/4286 or the mutant93/4286Ω*cssA* strains. At 48 h, brains were harvested and treated for histological analysis. Forty-five µm coronal sections were stained with cresyl violet. Overview (mosaic reconstruction from 10x individual pictures) of the hippocampal region of a representative animal infected with 93/4286 (A) or 93/4286Ω*cssA* (B). Overview (objective 20x) of the meninges (black arrowheads) and an inflamed penetrating vessel (white arrowheads) in an animal infected with 93/4286 (C). Close up view (objective 40x) of the ventricular space of the animal infected with 93/4286 showing inflammatory cells (white arrowheads) and possible intraventricular haemorrhage (black star) (D). Close up views (objective 40x) of the *corpus callosum* of animals infected with 93/4286 (E) or 93/4286Ω*cssA*, with the presence of infiltrated inflammatory cells (white arrowheads) (F).

838 **FIG 5.** Immunofluorescence analysis of brain sections of mice infected with wild type 93/4286 or
839 mutant 93/4286Ω*cssA* strains. Mice were infected and sacrificed as described in Fig. 4. Ten µm
840 brain sections were treated with a rabbit meningococcal antiserum and then a goat anti-rabbit Cy3
841 serum. Slides were counterstained with DAPI and observed using a Zeiss fluorescence microscope.
842 Overview of the hippocampal region of two representative animals infected with either the wild
843 type 93/4286 (A-C) or the mutant 93/4286Ω*cssA* (D-F) strains. In the insets (20X), *corpus callosum*
844 (B, E) and ventricles (C, F) from the brain of mice challenged with 93/4286 (B, C) or
845 93/4286Ω*cssA* (E, F). Large quantities of bacteria are detected in samples from mice infected with
846 the wild type strain. In red, *N. meningitidis* immunostained with meningococcal antiserum. In blue,
847 DAPI.
848
849 **FIG 6**. Cerebral bleeding in mice infected with 93/4286 or 93/4286ΩcssA strains. BALB/c mice
850 were infected with either the wild type strain 93/4286 (n=8) or the mutant strain 93/4286ΩcssA
851 (n=8) and sacrificed at 48 h. Brains were collected and immediately frozen in dry ice. Hemispheres
852 were cut in 30 µm cryosections and photographed to determine the number of hemorrhagic spots
853 and the areas of bleeding. Macroscopical assessment of cerebral haemorrhages in animals
854 challenged with the wild type or the mutant strain (A). Enumeration of bleeding spots (B) and
855 measurement of hemorrhagic areas (C) were carried out on 5 comparable brain sections/mouse.
856 Data are represented as mean ± SD. Differences were assessed by the Mann-Whitney test (*,
857 $P<0.05$).