A regulatory RNA is associated to invasive meningococcal disease in Europe

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Abstract
The strictly human pathogen Neisseria meningitidis is a commensal bacterium but can occasionally turn lethal causing septicaemia and meningitis. The mechanisms of how the meningococcus shifts to invasive infection remain poorly understood. Here we demonstrate that an eight-base pair tandem repeat deletion in the 5´-untranslated region of the polysaccharide capsule biosynthesis operon results in a hypercapsulation phenotype in clinical isolates. The increased capsule production significantly improves the bacterium survival in human serum while impairing its ability to adhere and colonise human pharyngeal cells. Among 4501 reported meningococcal cases in Europe from 2010-2018, the loss of an eight base-pair tandem repeat is three times more prevalent in invasive isolates (16.3%) compared to carrier isolates (5.1%). Combined results indicate that polymorphisms in this regulatory RNA contributes to meningococcal virulence.

Importance In this study we report a regulatory RNA to be directly involved in clinical manifestation of meningococcal disease. Using readily accessible WGS of meningococcus, we have now demonstrated that regulatory RNAs directly contribute to the progression of invasive meningococcal infection. We believe this novel combination of molecular and comparative regulatory RNA study could be used for the identification of additional RNAs involved in not only meningococcus but also pave the way for similar studies in other important bacterial pathogens. The identification of specific regulatory RNAs will no doubt facilitate clinicians, microbiologists, and public health practitioners to adjust their diagnostic techniques and treatments to best fit the condition of the patients.

Keywords: sRNA, tandem repeats, polysaccharide capsule, Neisseria meningitidis, meningococcal disease

Introduction
Neisseria meningitidis is a Gram-negative diplococcus and an obligate human commensal bacterium, which resides exclusively on the epithelium of the nasopharynx. Carriage is age-dependent and rises from 4.5% in infants (0-1 years) to 23.7% in adolescents (15-19 years) and down to 7.8% in middle aged adults (50 years) (1). By mechanisms not fully understood, the harmless colonisation can rapidly turn into an invasive infection leading to lethal septicaemia and meningitis. The incidence of invasive meningococcal disease is highly dependent on geographical region and season, and ranges from 0.1/100,000/population/year to almost 100/100,000/population/year (2). People with increased risk for invasive meningococcal infections are infants, adolescents and immunocompromised individuals (3-5).

Currently, there are twelve known serogroups of N. meningitidis identified based on the composition of their capsular polysaccharide (A, B, C, E – H, I – K, L – W, X, Y, Z) with six of these responsible for outbreaks; A, B, C, W, X, and Y (6). The capsular synthesis (cps) locus has been characterised for a wide range of serogroups and shows gene order synteny (6). In addition to serogroup, meningococci can be grouped into clonal complexes (cc) and sequence types (ST) by multilocus sequence typing (MLST) based on polymorphisms in housekeeping genes (2). In this study, we investigated meningococcal isolates from two Swedish adolescents who contracted meningococci after attending a ski trip to the French Alps in March 2017. During the trip one adolescent contracted invasive meningococcaemia and recovered. Two months later after returning home to Sweden, another adolescent succumbed to invasive meningococcaemia and a third adolescent was identified as an asymptomatic carrier. This study aims to elucidate and compare virulence mechanisms underlying the cause for the fatal meningococcal infection especially in the regulation of its polysaccharide capsule production. Using findings from the Swedish isolates, the subsequent work encompasses the investigation of correlations in the manifestation of diseases between 4501 meningococcal invasive- and carrier isolates from Europe.

Results
We whole-genome sequenced (WGS) both the Swedish meningococcal isolates used in this study and deposited them in the PubMLST database under following IDs; 17-264: 53777 (invasive isolate) and 17-271: 53778 (carrier isolate). Both isolates were identified to belong to serogroup C, cc32 and with identical typing genes as the French isolate (Table 1). Bacterial growth, micro- and macroscopical examinations revealed no significant difference between the isolates (Fig S1 and S2). Loci similarity comparison from the WGS encompassing 2219 loci revealed 98.3% (2181) loci conservation (Table EV2). The 1.7% (38) loci similarity comparison from the WGS encompassing 2219 loci revealed 98.3% (2181) loci conservation (Table EV2). The 1.7% (38) different loci between the isolates comprise of incomplete IS1655 transposase insertions in the 17-271 isolate, together with genes coding for outer membrane proteins, efflux pumps, and metabolic proteins (Table 3). Further analyses revealed that many hits of the variable loci were due to single nucleotide polymorphisms (SNPs) that were either true SNPs or sequencing errors. All true SNPs identified were found to generate the same gene products in both isolates. In addition, other hits were also later identified as sequencing error due to long Poly-G or Poly-C regions present over the locus.
Table 1. Typing output of 17-264 and 17-271 isolates recovered from PubMLST as well as the typing of the isolate responsible for the infection in the French Alps.

| ID     | Isolate | Country | Year | Disease | Species   | Capsule group | MLST  | Finetyping antigens |
|--------|---------|---------|------|---------|-----------|---------------|-------|-------------------|
| Not available | Not available | France | 2017 | Invasive | Neisseria meningitidis | C | 32 | ST-32 complex | 7 | 16-29 | F3-3 |
| 53777  | 17-264  | Sweden  | 2017 | Invasive | Neisseria meningitidis | C | 32 | ST-32 complex | 7 | 16-29 | F3-3 |
| 53778  | 17-271  | Sweden  | 2017 | Carrier  | Neisseria meningitidis | C | 32 | ST-32 complex | 7 | 16-29 | F3-3 |

The final five hits confirmed as different are NEIS0210 (pilE), IGR\textsuperscript{\textregistered}NEIS0055 (5′-UTR-cssA), NEIS0568 (pglE), NEIS1310 (modA12), and NEIS2986 (hypothetical protein) (Highlighted in Table EV3). The pilE gene encodes for the major subunit of Type-IV pilus (T4P) and is different due to antigenic variation, usually observed even within the same clonal population (\textsuperscript{8}). The 5′-untranslated region (UTR) of the capsular biosynthesis operon cssA of the invasive isolate 17-264 lacks an eight base pair (8bp) tandem repeat (5′-TATACTTA-3′). The pglE gene is involved in the glycosylation of T4P and is known for its ON/OFF phase-variation depending on heptarepeats (5′-CAACACAA-3′) (\textsuperscript{9}). The two isolates have different transcriptional pglE frames due to different amount of heptarepeats but both result in the same OFF phase. Similar to the regulation to pglE, the modA12 gene encoding a methyltransferase of DNA is regulated by tetrarepeats, 5′-AGCC-3′ (\textsuperscript{10}) and although both isolates have different amounts of repeats, they are both in the same OFF phase. NEIS2986 is a hypothetical protein of 90 amino acid, with no elucidated function and no recognised protein motifs. The predicted protein sequence does not contain start codon, which indicate that this is a pseudogene. Overall, among the 38 loci identified as different, the only \textit{bona fide} hit is the 5′-UTR-cssA between the two isolates.

Through immunoblot analysis, we reveal that the invasive isolate 17-264 has a higher expression of CsaA (capsular synthesis protein), PilE (predominant minor pilin in the T4P involved in adhesion), and Opa, (opacity proteins involved in adhesion) (Fig 1A). Expression of other virulence factors such as fHbp, (responsible for sequestering immune factor H), Hfq (RNA-chaperone important for sRNA-mediated gene regulation), PorA (voltage-gated, cation channel) RmpM (periplasmic protein interacting and stabilising PorA and PorB), and other pilin proteins (pilus and pilus-associated proteins that form the T4P) remain same for both isolates and were used as controls. The capsular polysaccharide cssA coding region was also initially annotated as different between the isolates (Table EV3), but further examination reveals the presence of a single base polymorphism but coding for the same amino acid and therefore does not explain the higher expression of CsaA in invasive isolate 17-264 compared to carrier isolate 17-271. These observations prompted us to further investigate into the regulatory region of the capsular polysaccharide operon.

The WGS data reveal the loss of an 8bp tandem repeat in the 5′-UTR-cssA region of the invasive isolate 17-264 compared to the carrier isolate 17-271 (Fig 1B). Secondary structure predictions of the 5′-UTR-cssA mRNA confirms a possible stem-loop formation in the carrier isolate 17-271 (\textit{ΔG}: -15.60 kcal/mol), while the invasive isolate 17-264 possesses a thermodynamically less stable (\textit{ΔG}: -11.30 kcal/mol) secondary structure (Fig 1C). Previously, we identified that the 5′-UTR-cssA, functions as an RNA-thermosensor, controlling the expression of CsaA in a temperature dependent manner (\textsuperscript{11}). We therefore investigated temperature dependent expression of CsaA in these two clinical isolates. Results show a temperature-mediated upregulation of CsaA in the 17-264 whereas the level of CsaA in the 17-271 remained low (Fig 1D - top). Since a \textit{bona fide} RNA-thermosensor should be able to function independently of the native bacterial host factors, an ectopic bacterial host (\textit{Escherichia coli}) was selected to further investigate this thermal regulation. The respective 5′-UTR-cssA from each isolate was introduced upstream of a green fluorescent reporter EGFP in the pEGFP-N2 vector and transformed into \textit{E. coli}. Immunoblot results from \textit{E. coli} were consistent with their respective meningococcal background (Fig 1D – lower).

Table 2. Summary of loci comparison between 17-264 and 17-271 isolates. Missing-, and incomplete loci in both isolates were excluded from calculations.

| Matching loci | Missing loci | Different loci | Incomplete loci | Frequency matching (%) |
|---------------|--------------|----------------|-----------------|-----------------------|
| 2181          | 763          | 38             | 60              | 98.3                  |
Table 3. List of the 38 different loci in the WGS comparison between 17-264 and 17-271.

| Locus           | Product          | Cause/Effect                                                                 |
|-----------------|------------------|-------------------------------------------------------------------------------|
| NEIS0210        | pilE             | Antigenic variation                                                          |
| NEIS0568        | pglE             | 7bp tandem repeats (17-264: 11 and 17-271: 13), different frame but both off phase |
| NEIS2986        | hypothetical protein |                                                                  |
| igr_up_NEIS0055 | 5’-UTR-cssA      | 8bp tandem repeat missing in 17-264                                           |
| NEIS1310        | modA12           | 4bp tandem repeats (17-264: 14 and 17-271: 23), same frame and both off phase |
| NEIS0033        | pilC2            | SNP                                                                           |
| NEIS0054        | cssA             | End of contig* in 17-271 sequencing but otherwise identical                  |
| NEIS0085        | protein export protein |                                               |
| NEIS0213        | pglA             | SNP/undecided nucleotide in sequencing                                        |
| NEIS0297        | insertion element IS1655 transposase | Incomplete transposase element in 17-271                                      |
| NEIS0377        | putative integral membrane protein | SNP/undecided nucleotide in sequencing                                         |
| NEIS0380        | pglL             | End of contig* in 17-264 sequencing but otherwise identical                  |
| NEIS0402        | pglF             | End of contig* in 17-264 sequencing but otherwise identical                  |
| NEIS0493        | hypothetical protein | 5’ sequencing error, undecided nucleotides in sequencing                      |
| NEIS0648        | ksgA             | SNP/undecided nucleotide in sequencing                                        |
| NEIS0710        | pnp              | SNP                                                                           |
| NEIS0715        | cysteine synthase | SNP                                                                           |
| NEIS0904        | transposase for IS1655 | Incomplete transposase element in 17-271                                    |
| NEIS0976        | transposase for IS1655 | Incomplete transposase element in 17-271                                    |
| NEIS1008        | transposase for IS1655 | Incomplete transposase element in 17-271                                    |
| NEIS1055        | transposase for IS1655 | Incomplete transposase element in 17-271                                    |
| NEIS1085        | mpl              | SNP                                                                           |
| NEIS1156        | hypothetical protein | SNP, Poly-G region sequencing error                                    |
| NEIS1288        | putative aldehyde dehydrogenase | SNP and end of contig*                                                      |
| NEIS1381        | transposase for IS1655 | Incomplete transposase element in 17-271                                    |
| NEIS1418        | putative membrane peptidase | Sequencing error                                                            |
| NEIS1852        | farA             | Sequencing error and end of contig*                                         |
| NEIS1853        | farB             | End of contig*                                                               |
| NEIS1884        | transposase for IS1655 | Incomplete transposase element in 17-271                                    |
| NEIS1902        | lgtA             | Poly-G region sequencing error and end of contig*                            |
| NEIS1926        | putative inner membrane protein | End of contig*                                                              |
| NEIS1943        | nalP             | SNP, Poly-C region sequencing error in 17-271                               |
| NEIS1993        | insertion element IS1655 transposase | Incomplete transposase element in 17-271                                    |
| NEIS2099        | putative immunity protein | Sequencing error and end of contig*                                        |
| NEIS2124        | lipoprotein      | End of contig*                                                               |
| NEIS2125        | insertion element IS1655 transposase | Incomplete transposase element in 17-271                                    |
| NEIS2155        | lgtD             | Poly-G region sequencing error and end of contig*                            |
| igr_up_NEIS1364 | porA promoter    | SNP in 17-271. WB shows no difference                                        |

*End of contig refers to sequencing contig is incomplete in covering the loci in one WGS sample and therefore are in the list as different. Unless stated otherwise the sequencing is identical up to that point.*
An elevated expression of the CssA protein is however not definite evidence that more surface capsule is produced. To address this, “dot blots” assays were performed, as it has previously been used to semi-quantitatively measure meningococcal capsule production (12-14). Dot blots using serogroup C specific anti- sera show that surface polysaccharide capsule is produced in high amounts regardless of
cells, Detroit 562 were performed. Results reveal that the less capsulated carrier 17-271 isolate has a significant, ten times more effective adherence to the cells compared to the hypercapsulated invasive 17-264 isolate (Fig 2B).

These findings from the Swedish isolates prompted the question on growth temperature in the invasive isolate 17-264, while the carrier isolate 17-271 retain a temperature dependent production of capsule (Fig 1E – top). A fraction of the polysaccharide that are the constituents of the capsule are known to be released or “shed” into the surrounding environment by meningococci, when not recycled by the bacterium (15). The increased capsule expression in 17-264 is also evident when observing greater amount of shed capsule from liquid growth at 37°C compared to the 17-271 (Fig 1E – bottom).

To test if the hypercapsulation will improve bacterial survival when facing human immune factors, the two meningococcal isolates were subjected to serum killing assay using pooled serum from healthy individuals. Exposing bacteria to different serum concentrations revealed a trend of higher survival for the invasive isolate 17-264 compared to the carrier isolate 17-271 (Fig 2A – left). The difference becomes evident after 40 minutes of serum exposure, where the carrier isolate 17-271 was not able to resist 40 minutes serum exposure effectively in any higher serum concentration than 5%, while the invasive isolate 17-264 is able to survive in all serum concentrations, as well as growing more than the input colony forming unit (cfu) (Fig 2A - right). The hypercapsulation phenotype allows the bacteria to resist complement serum killing, however capsulation could inadvertently affect the ability of the bacteria to interact and adhere to pharyngeal cells due to inaccessible surface protein masked by the capsule (16). To investigate this, adhesion assays on human pharyngeal

![Fig. 1. 5´-UTR-cssA mapping, virulence factor characterisation, and temperature-mediated expression of CssA and capsule A) Immunoblot panel display the expression level of a variety of virulence related- and house-keeping proteins. Highlighted in bold are the virulence proteins upregulated in the invasive isolate 17-264; CssA, Opa and PIIIE. B) Illustration showing the ctr and cps loci with focus on the intergenic region. The 5´-UTR-cssA sequences from the two clinical isolates were aligned. An 8bp deletion was observed in the invasive isolate. C) Secondary structure prediction of the 5´-UTR-cssA RNA using VARNA14 illustrating the thermodynamic stable stem-loop structure of the carrier isolate 17-271 (∆G: -15.60 kcal/mol) and a smaller and less stable stem-loop of the invasive isolate 17-262 (∆G: -11.30 kcal/mol). The tandem repeat, RBS and start codon are marked in red/green, cyan and yellow, respectively. Small blue dots designate base pairing. D) Immunoblots of temperature mediated expression shows an increasing and constitutive high expression of CssA in the invasive isolate 17-264 compared to carrier isolate 17-271 (top). The 8bp tandem repeats function ectopically, controlling EGFP expression similar to their respective meningococcal background. (lower). E) Dot blots of capsular production on the surface of the bacteria show a constitutively high capsule production in the invasive isolate 17-264 compared to carrier isolate 17-271 (top). Serial dilution of supernatant from liquid culture grown at 37°C shows that the invasive isolate 17-264 sheds more capsule into the medium compared to the carrier isolate 17-271 (lower). Data information: All blots are representative of at least three biological replicates.
The majority of the European invasive isolates investigated in this study are of serogroup B, with a higher distribution of a single 8bp tandem repeat (25.2%, n=2343) than the other serogroups respectively, C (12.3%, n=277), Y (5.5%, n=642), W (2.5%, n=937) and in non groupable (NG) (20.2%, n=94) (Fig 3B). As specific ccs are more prevalent in invasive disease, the configurations of 5’-UTR-cssA (27.7%) of invasive isolates were also divided into respective complexes (Fig 3C). The same cc (cc32) as the investigated clinical isolates used in this study showed an even higher frequency with the loss of an 8bp repeat, 27.8% (n=213), than the overall European invasive isolates of 16.3%. The study showed an even higher frequency with the loss of an 8bp tandem repeat, 27.8% (n=213), than the overall European invasive isolates of 16.3%. The carrier isolate 17-271 is approximately 10 times better in adherence compared with the invasive isolate 17-264, p=0.045. Data information: All graphs are presented with mean ± SEM. *P<0.05 (Student’s t-test).

Discussion

Our work supports a regulatory RNA to be directly involved in the clinical manifestation of meningococcal disease. Epidemiological analysis of 4501 meningococcal isolates reveal that the loss of an 8bp tandem repeat in this regulatory RNA is more than three times frequent in invasive clinical isolates thus emphasising its role in virulence. Molecular analyses from the two Swedish isolates in this study have further confirmed our previous finding that the 8bp tandem repeats in the 5’-UTR-cssA regulate CssA expression in a temperature dependent manner (11). In addition, we demonstrated that the higher expression of CssA leads to higher production of capsule as shown by the dot blot assays.

It is known from previous studies that both expression levels of T4P and capsule affects the ability of meningococci to adhere to human cells (17, 18). Our results indicate that while the invasive isolate 17-264 express more PilE protein, the most abundant minor pilin in the makeup of the T4P it still has a reduced ability to adhere to cells. Our findings supports previous research that the thicker polysaccharide capsule could interfere with important surface structures such as T4P thus sequestering their function for adhesion to pharyngeal cells (19). Previous study has shown that T4P and OpA mediates endothelial interactions in a synergistic manner in capsulated strains (20). It is also known that expression of PilE and OpA is in unison to facilitate Neisserial microcolony formation (21).

Our analysis here also show nine copies of incomplete IS1655 elements in the carrier isolate, 17-271. All of the IS1655 hits were sequenced to the end of contigs in the WGS data and with a short alignment to the reported IS1655. This IS element is an exclusive feature of meningococcus and found in high numbers in several strains (22, 23).
To date, no work has been done to investigate whether IS1655 is involved in meningococcal pathogenesis, given its prominent and diverse presence across the meningococcal genomes.

In our previous study investigating this 8bp tandem repeats (11), we speculated that the three configurations of the 8bp tandem repeats are an evolutionary step-by-step process. The native two 8bp tandem repeats and temperature regulated capsule expression are beneficial for the bacterium during non-invasive colonisation of the host nasopharynx. Meningococci face environment switches constantly, such as localised inflammation caused by the bacterium itself, other microbes, or by entering the blood circulation. It is our hypothesis that the stress induced by high amounts of immune factors together with a febrile condition will make the thermostable two 8bp repeats superfluous, as the need for protection by expressing more polysaccharide capsules is great. This selection pressure induced by the host alters the meningococcal population through the loss of an 8bp repeat in the 5′-UTR-cssA to produce more capsule constitutively. The rapid onset of the invasive disease together with the presence of all three different 5′-UTR-cssA thermosensors in carrier isolates suggest this selection to take place in the nasopharynx. To investigate the loss of 8bp mechanism, we subjected the carrier isolate 17-271 to a sub-lethal bacterial selection strategy to the new environment (26). During recovery conditions after stress, the meningococci would shift towards a population that restores colonisation by reducing capsule expression with a reinstated thermostable 5′-UTR-cssA by one or two point mutations to strengthen the stem-loop structure (Fig S4). Interestingly, among the 4501 meningococcal isolates, we could observe this phenomenon as an almost two-fold higher frequency of one 8bp tandem repeat with substitutionary mutations among the carrier isolates compared to the invasive isolates. Capsular switching is a phenomenon where meningococci acquire parts of the csr locus from other serogroups such as B to C or the reverse (27). The serogroup C, cc-32 isolates used in this study could support a capsule switching event prior to the alteration of the 5′-UTR-cssA as similar capsular switching has been reported previously (28, 29). It is interesting to hypothesise how capsular switching could also contribute to the “reversal” of a 1x8bp or 1x8bp + 2sub 5′-UTR-cssA to a “natie” 2x8bp 5′-UTR-cssA as a consequence of capsular switching bringing the 5′-UTR-cssA of the “donor” DNA, however previous work has shown the intergenic region between ctrA and cssA is not included in capsular switching (22).

While it is difficult to determine all the host genetic- and environment factors involved, we postulate that the loss of an 8bp tandem repeat hypercapsulation phenotype of the 17-264 isolate contributed to the fatal disease manifestation in the adolescent girl from Sweden. Statistical analysis further strengthen this hypothesis as the one 8bp tandem repeat is three-fold more frequent among invasive isolates compared to the carrier isolates. Our epidemiology data also show the prevalence of the loss of an 8bp tandem repeat is most prominent in Serogroup B, cc32. In addition we demonstrated that invasive isolates of other cc such as cc41/44 regulate CsaA expression solely depending on the configuration of the 8bp tandem repeats (Fig S5) regardless of cc. It is important to note that the isolation of clinical isolates are from two distinct sites; the nasopharynx (carrier) and blood/cerebrospinal fluid (CSF) (invasive). The presence of all three configurations of the 8bp tandem repeats in both sites reflect the importance of this regulatory RNA in controlling its capsular biosynthesis in both environments. However, the significant higher prevalence of the hypercapsulated single 8bp configuration observed among the invasive isolates found in blood/CSF would be beneficial for the bacteria to evade the host immune killing. It is hard not speculate that while the single 8bp background could be due to isolation sites, our statistics finding emphasize that this regulatory RNA plays a pivotal role in virulence and is associated meningococcal disease manifestation.

Due to the lack of suitable infection models, we believe that molecular comparative WGS between meningococcal isolates from invasive and carrier cases is the best alternative. With WGS, polymorphism comparisons should include regions such as 5′-UTRs and regulatory RNAs as they are significantly involved in the expression of many virulence factors exemplified here by capsule production. Comparative WGS will facilitate future studies into underlying mechanisms involved in disease manifestation, improve diagnostic techniques and treatments by clinicians and advance future vaccine development.

**Materialsand Methods**

**Bacterial isolates and DNA extraction.**

The bacteria were cultured overnight on chocolate agar in 37°C and 5% CO₂, serogrouped by coagglutination, and stored at −70°C. In this study, both meningococcal isolates were isolated during May 2017 and whole-genome sequencing (WGS) was performed. DNA was extracted using a customized protocol and QIAasympath DSP virus/pathogen Midi kit on the QiAasympath system (Qiagen, Hilden, Germany). WGS was performed using the Nextera XT DNA library preparation kit, the MiSeq reagent kit v3, 600 cycles on the Illumina MiSeq platform (Illumina, San Diego, CA). The reads were assembled de novo using Velvet (30). The quality of sequencing was controlled by the N50 value, contig count and coverage. The sequences were trimmed until the average base quality (Phred score) was >30 in a window of 20 bases. The assemblies were uploaded to the Neisseria PubMLST database, and the sequences were automatically scanned and tagged against defined “NEIS” loci in the database (31). Information regarding the typing of the French isolate was obtained through personal communication with Dr. Muhamed-Kheir Taha, Institut Pasteur, Paris.

**Bacterial culturing**

For all other purposes, bacteria were grown with Brain Heart Infusion (BHI) broth (Sigma-Aldrich, Saint-Louis, MO) or agar supplemented with horse serum (Thermo Fisher Scientific, Waltham, MA). For all experiments, bacteria were inoculated on BHI agar for 16h, in 37°C and 5% CO₂. Liquid cultures were grown in BHI broth starting at an optical density at 600 nm (OD₆₀₀) of 0.05 and cultured to OD₆₀₀:0.6 where protein or DNA were harvested.

**Scanning Electron Microscopy**

Bacteria were fixed with 6% paraformaldehyde and transferred to a pre-sputtered filter (Polymix, NL 16, GE Healthcare UK Limited, Buckinghamshire, UK), rinsed in distilled water and placed in 70% ethanol for 10 minutes, 95% ethanol for 10 minutes and absolute ethanol for 15 minutes, all at 4°C and then into acetone. Specimens were then dried using a critical point dryer (Balzer, CPD 010, Lichtenstein) with CO₂. After drying, filter was mounted on an aluminium stub and coated with Platinum (Q150T ES, West Sussex,
UK). The specimens were analysed in an Ultra 55 field emission scanning electron microscope (Zeiss, Oberkochen, Germany) at 5 kV.

**Immunoblotting and dot blotting**

For immunoblotting, bacterial pellet was spun down at 7,000 g for 10 minutes and lysed in Buffer A (200mM KCl, 50mM Tris-HCl pH 8.0, 1mM EDTA, 10% Glycerol). Cell debris was spun down at 12,000 g for 5 minutes and lysate collected for protein quantification. Using BCA (Thermo Fisher Scientific). SDS-loading buffer was added and boiled at 98°C for 10 minutes before protein was subjected to electrophoresis on 4-12% Bis-Tris gel (Thermo Fisher Scientific) and run at 110V. Gels were transferred to PVDF membranes using the Trans-Blot Turbo system (Bio-Rad, Hercules, CA). For dot blots, bacteria were harvested from plates grown at 30-, 37-, and 42°C, and supernatant was harvested from liquid growth at 37°C. Supernatant was sterile filtered and serial diluted in PBS while harvested bacteria were lysed as described previously and diluted 1x10⁸ in PBS. Amershams Hybond-P PVDF membranes (GE healthcare, Chicago, IL) were activated and 10µl of the samples were dotted onto the membrane and left to dry for one hour at 37°C. Membranes were blocked in 5% milk for one hour at room temperature (RT) and incubated with primary antibody/anti-sera in PBS + 0.1% Tween, for one hour at RT. After washing membrane four times 5 minutes, secondary antibody was added in PBS + 0.1% Tween for one hour RT. Membranes were exposed by Amershams ECL reagents (GE healthcare) and visualised by GelDoc XRS+ (Bio-Rad). Commercial antibodies used was anti-RecA, (Abcam, ab63797) (crossreacts with N. meningitidis RecA), anti-PorA P1.7, (NIBSC, 01/514) and anti-Flhbp, (NIBSC, 13/216). The remaining antibodies used are custom antibodies obtained from Yvonne Pannekoek (anti-Hfq), Hank Seifert (anti-PilO), Ann-Beth Jonsson (anti-PilC) and Ryoma Nakao (anti-Rnmp, PilE, Opa, CssA and GroEL).

**Human serum stress and killing assay**

Human serum killing assay was performed with bacteria resuspended in PBS and diluted to a concentration of 1x10⁵ CFU/ml in DMEM-medium (Gibco, Thermo Fisher Scientific). Serum from healthy humans (Sigma-Aldrich) was used to stress bacteria at 37°C in the presence of 5% CO2 for 20-, or 40 minutes (killing assay) or hours (serum stress induction). Survival of bacteria in the presence of sera was determined by dot-plating 10µl and counting CFUs after overnight incubation. The percentage of survival was measured relative to input.

**Bacterial adhesion assay**

Detroit 562 cells were cultured in DMEM, supplemented with 10% FBS (GE Healthcare). 5x10⁵ cells were seeded per bacterial isolate tested. Cells were washed with PBS and covered with 2mL DMEM without FBS. Bacteria were resuspended in PBS to an OD₆₀₀ of 0.5 and added to the cells at a MOI of 50. Bacteria and cells were incubated at 37°C at 5% CO₂ for one hour, and washed three times with PBS. Cells were lysed for 10 min with 1% saponin (Sigma-Aldrich), and the lysate was diluted 1:3 in PBS before dribble-plating and incubated at 37°C, ON and analysed the next day. CFU of the negative control (no cells) were subtracted from CFU of the output. The result was normalised to the CFU of the input.

**Whole-genome sequencing with loci comparison**

17-264 and 17-271 isolates were compared using the “Genome comparator” function in the PubMLST database. Sequence alignments were performed for all loci that were marked as different between the isolates. For further protein sequence prediction and alignment, online tools; ExPASy and Clustal Omega (SIB Swiss Institute of Bioinformatics, Lausanne, Switzerland) were used.

**5’-UTR-cssA mapping and in silico RNA structure predictions**

The PubMLST database was used for identifying meningococcal isolates and selected according to the following search criteria. The range from the 1st of January 2010 to 31st of December 2018 and geographic restriction to the continent of Europe. The searches were then separated by the designation of “carrier” or “invasive (unspecified/other)”. Hits were BLAST to a 200bp long sequence that encompasses the 5’UTR of the cssA gene and the beginning of the coding region of cssA. The results were exported and analysed by excluding all hits that had less than 100bp alignment to the search query. Any isolate that was suspect of non-canonical 5’UTR-csSA or where the sequence could not be retrieved, was excluded from the analysis. To predict, analyse, and visualise secondary structure of RNA, multiple available softwares were used. RNAfold by ViennaRNA package (32) and mFold (33) were used for structure prediction data and folding based on the minimum free energy. Varna (34) was used to visualise the secondary structure obtained together with annotated nucleotides.

**Statistical methods**

All experiments were performed with two or three biological replicates. Data from human serum killing assay and bacterial adhesion assay are shown with mean ± SEM, and students t-test was used to calculate statistical significance. Normal distribution are assumed for this data. The observational data of the 5’UTR-cssA mapping is shown with mean distribution and χ²-test was performed to calculate statistical significance in group distributions. P values of less than 0.05 was considered statistically significant. Graphpad Prism v5.04 (Graphpad software, Inc, La Jolla, CA) and SPSS v25 (IBM, Armonk, NY) was used to plot data and perform statistical analysis.

**End Matter**

**Author Contributions and Notes**

S.J. performed the meningococcal isolates isolation, typing and WGS. J.K., H.E. and E.L. performed the experiments and analysed the data. J.K. performed the European clinical data collection and analysis. E.L. provided overall direction. E.L and J.K. performed literature search and wrote the manuscript with input from H.E. and S.J. The authors declare no conflict of interest. This article contains supporting information online.

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