**LETTER**

**Temperature triggers immune evasion by Neisseria meningitidis**

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*Neisseria meningitidis* has several strategies to evade complement-mediated killing, and these contribute to its ability to cause septicaemic disease and meningitis. However, the meningococcus is primarily an obligate commensal of the human nasopharynx, and it is unclear why the bacterium has evolved exquisite mechanisms to avoid host immunity. Here we demonstrate that mechanisms of meningococcal immune evasion and resistance against complement increase in response to an increase in ambient temperature. We have identified three independent RNA thermosensors located in the 5′ untranslated regions of genes necessary for capsule biosynthesis, the expression of factor H binding protein, and sialylation of lipopolysaccharide, which are essential for meningococcal resistance against immune killing2. Therefore increased temperature (which occurs during inflammation) acts as a ‘danger signal’ for the meningococcus, enhancing its defence against human immune killing. Infection with viral pathogens, such as influenza, leads to inflammation in the nasopharynx with an increased temperature and recruitment of immune effectors3,4. Thermoregulation of immune defence could offer an adaptive advantage to the meningococcus during co-infection with other pathogens, and promote the emergence of virulence in an otherwise commensal bacterium.

*Neisseria meningitidis* is an obligate human pathogen and important cause of sepsis and meningitis5, with peaks of disease often preceded by influenza outbreaks in temperate climates5. The bacterium has evolved exquisite mechanisms to evade immune responses5, including expression of a polysaccharide capsule (containing sialic acid in serogroup B, C, Y and W strains)6,7, sialylation of lipopolysaccharide, and recruitment of the human complement regulator factor H, via high-affinity interactions with bacterial factor H binding protein8,9. The reasons why such mechanisms have evolved in an otherwise commensal bacterium is uncertain as systemic infection represents an evolutionary dead end.

Previously we found that resistance of the meningococcus against complement-mediated killing is enhanced after insertion of the mobile element IS**_{301}** into the 134-base-pair (bp) intergenic region (IGR) between the *css* (encoding capsule biosynthesis) and *ctr* (capsule export) operons in the capsule biosynthesis locus (*cps*). To identify other changes modulating serum resistance, we subjected the *N. meningitidis* strain *S3* (ref. 11) to serial passage in 6% human serum; within six rounds, *S3* became resistant to complement-mediated killing as a strain with *IS**_{301}** in the IGR (Supplementary Fig. 1). We further characterized six passaged strains (selected serum resistant, SSR1–6) that were more resistant to complement than *S3* (Fig. 1a). Resistance did not result from insertion of *IS**_{301}**; instead five strains (all except SSR2) had lost a single copy of a duplicated 8-nucleotide sequence (TATACTTA) located 15 nucleotides upstream of the *cssA* start codon in the 5′ untranslated region (5′ UTR) of *css* messenger RNA (Fig. 1b, c and Supplementary Fig. 2). We have increased levels of *cssA*, which catalyses an early step in capsule biosynthesis12. Comparison of *S3* with SSR, and isogenic strains (both containing an antibiotic-resistance cassette downstream of the *css* operon) with one (Δ8) or two (wild-type) copies of the 8-bp sequence demonstrates that loss of 8 bp causes increased *CssA* levels and capsule expression (Fig. 1d, e). The increased serum resistance of SSR2 resulted from reduced levels of PorA (Fig. 1b), a target of bactericidal antibodies13.

To define how the 8-bp sequence contributes to capsule expression, we performed DNA footprinting of the IGR with integration host factor (IHF) and factor for inversion stimulation (FIS). Despite potential recognition sites for these proteins13,14, there was no difference in IHF or FIS binding to the wild-type and Δ8 IGRs (Supplementary Fig. 3).

Analysis of translational reporters in the *N. meningitidis* *cps* demonstrated that loss of 8 bp leads to a marked increase in reporter activity of *css* but not *ctr* (Supplementary Figs 4 and 5), demonstrating that Δ8 influences the capsule biosynthesis operon. Furthermore, significantly increased *css* reporter activity with the Δ8 IGR was evident in 38 transcription factor mutants (Supplementary Table 1 and Supplementary Fig. 5), and northern blot analysis demonstrated that *css* mRNA levels are unaffected by the number of copies of the 8 bp (Fig. 1f). Therefore, the 8-bp sequence affects *CssA* post-translationally.

We noticed that the *css* 5′ UTR is predicted to form a stem–loop structure that includes the ribosome-binding site (RBS) (Fig. 2a), consistent with an RNA thermosensor15. In RNA thermosensors, the transcript assumes a hairpin structure at lower temperatures that occludes the RBS, and stalls protein translation; higher temperatures destabilize the secondary structure which allows translation. The Δ8 mRNA, on the other hand, is predicted to form a limited stem–loop with a single-stranded region by the RBS (Fig. 2a). Consistent with a thermosensor, *CssA* levels increase in *N. meningitidis* grown at increasing temperatures (Fig. 2b). By contrast, loss of 8 bp leads to increased *CssA* levels at lower temperatures and less pronounced increase at higher temperatures, suggesting that this change disrupts the thermosensor and dysregulates capsule biosynthesis.

RNA thermosensors should function in a heterologous host. Similar to *N. meningitidis*, thermal regulation of *CssA* was evident in *Escherichia coli* containing *CssA* and the wild-type IGR on a plasmid; *CssA* expression was dysregulated with the Δ8 IGR (Fig. 2c). Additionally, *in vitro* transcription/translation assays demonstrated that *CssA* synthesis increased with an increase in temperature and on loss of one copy of the 8-bp sequence (Fig. 2d) in the absence of any transcription factor. Furthermore, we introduced nucleotide changes into the 5′ UTR predicted to alter the stability of the thermosensor, including substitutions at the same position (+92_UcG or +92_UcG, Fig. 2a) expected to have opposing effects. Expression of *CssA* from plasmids containing these changes was consistent with a thermosensor in the 5′ UTR (Fig. 2e). We also performed RNA toe-printing at 30 °C, 37 °C and 42 °C to assess binding of ribosomes to the nascent *css* transcript. The results demonstrate that ribosome binding is enhanced to mRNA from the Δ8 compared to the wild-type 5′ UTR, with differences most marked at 30 °C (Supplementary Fig. 6). Finally we found that thermoregulation of *CssA* is

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evident in strains of different capsular serogroups and hypervirulent lineages (Fig. 2f). Together, these results confirm the presence of an RNA thermosensor controlling the capsule biosynthesis operon in \textit{N. meningitidis} across a range of strains.

Previous reports of RNA thermosensors in pathogens are restricted to facultative organisms and govern transcription factors that mediate transition of bacteria from external to internal environments. As the meningococcus is an obligate commensal residing in the nasopharynx, we compared the dynamic response of the canonical PrfA \textit{Listeria} thermosensor with the meningococcal Css thermosensor (which directs capsule biosynthesis) across a range of strains. The 134-bp css/ctr intergenic region showing the ribosome-binding site (RBS), transcriptional start sites (Fig. 1c), and open reading frames, and location of the duplicated 8-bp sequence. Resistant strains exhibit increased capsule expression by FACS. MFI, mean fluorescence index. e. CssA expression in isogenic strains with one or two copies of the 8-bp sequence. f. Northern blot analysis demonstrates that increased CssA levels are not associated with increased cssA mRNA, tmRNA, transfer-messenger RNA. Findings were confirmed in three biological replicates. Error bars show s.d. of experiments performed in triplicate.

Next we determined the prevalence of IGR polymorphisms in 265 meningococcal disease isolates. The wild-type sequence (two copies of 8 nucleotides) was most frequently found (201 of 265, 75.8%); of note, no polymorphism was detected in the 5 UTR. We performed DNA sequencing modification (shown in Fig. 3a) grown at temperatures indicated; the predicted effects shown. f. Thermoregulation of CssA is evident in \textit{N. meningitidis} across different capsular serogroups and lineages. cc, clonal complex. Findings were confirmed with three biological replicates.

Figure 1 | Loss of 8 bp increases capsule expression and complement resistance. a, b. Selected serum resistant (SSR1–6) strains demonstrate enhanced resistance in human serum against complement-mediated killing (a), and increased expression of CssA (b, except SSR2: RecA loading control) compared with the parental strain, S3, c. The 134-bp css/ctr intergenic region showing the ribosome-binding site (RBS), transcriptional start sites (Fig. 1c), and open reading frames, and location of the duplicated 8-bp sequence. d. Resistant strains exhibit increased capsule expression by FACS. MFI, mean fluorescence index. e. CssA expression in isogenic strains with one or two copies of the 8-bp sequence. f. Northern blot analysis demonstrates that increased CssA levels are not associated with increased cssA mRNA, tmRNA, transfer-messenger RNA. Findings were confirmed in three biological replicates. Error bars show s.d. of experiments performed in triplicate.

in most instances (50 out of 64 isolates, 78.1%). Only a few strains (8 out of 64, 12.5%) had the Δ8 sequence with no changes, and the single, TATGCTTAT polymorphism (Δ8AG/G) was present in the other strains (6 out of 64, 9.4%), whereas TATACCTAT (Δ8TG/C) was never detected. We analysed the effect of these polymorphisms in \textit{E. coli} plasmid reporters (Fig. 3d), and found that whereas Δ8AG partially restores CsaA thermal regulation after deletion of 8 nucleotides, Δ8AT/GC re-establishes thermosensing (consistent with its predicted structure and RNA toe-printing; Supplementary Figs 6 and 7). By contrast, Δ8TG/C (which was never found) leads to markedly increased CsaA expression at all temperatures. Of note, the compensatory polymorphisms occur at similar frequencies in different clonal complexes of the meningococcus, consistent with them arising on several occasions.

The conservation of the wild-type sequence and prevalence of compensatory polymorphisms emphasize the importance of capsule thermoregulation in the meningococcus. Hence, we examined whether other factors involved in immune escape are subject to similar regulation. Of note, expression of factor H binding protein (which recruits the host complement regulator factor H) and Lst (necessary for lipopolysaccharide sialylation) also increase with increasing temperature (Fig. 3e), in contrast to proteins not involved in immune escape (such as PorB, Pilin, RmpM and RecA), which are unaffected by temperature (Fig. 3f). To define the mechanism of thermosensing of fHbp and Lst,
we analysed E. coli plasmids containing these genes and observed thermal regulation (Fig. 4a). Furthermore, thermoregulation of these proteins in N. meningitidis was independent of 38 transcription factors (Supplementary Fig. 8), and thermoregulation of fHbp and Lst was detected in both in vitro transcription/translation assays (with fixed amounts of DNA) and in vitro translation assays (with fixed amounts of RNA; Fig. 4a), indicating that the 5’ UTRs of fHbp and Lst contain RNA thermosensors, consistent with secondary RNA structure predictions (Supplementary Fig. 9).

Temperature therefore acts as a danger signal for the meningococcus, prompting the bacterium to enhance expression mechanisms of immune evasion via three independent thermosensors dedicated to single proteins or pathways. To determine the influence of temperature on meningococcal complement resistance, bacteria were grown at 30 ºC then incubated at 30 ºC or 37 ºC for one hour. Bacteria that had been equilibrated at the increased temperature expressed more CsaA, fHbp and Lst (Supplementary Fig. 10) and were significantly more resistant against complement than those left at 30 ºC (Fig. 4b), demonstrating that thermal regulation of immune defence mechanisms has a marked effect on bacterial survival in the presence of human complement.

An increase in temperature is a cardinal feature of inflammation, which is associated with extravasation of serum components and recruitment of phagocytes. In these circumstances, increasing expression of factors necessary for immune evasion would provide a considerable adaptive advantage to a microbe in the nasopharynx (Fig. 4c). Influenza provokes complement activation in the upper airway3, a rise in core temperature and recruitment of innate immune effectors. Thermoregulation of microbial defence mechanisms would prevent bystander killing and enhance systemic dissemination to warmer body sites that are replete with immune effectors.
METHODS SUMMARY

N. meningitidis and E. coli were grown on brain heart infusion (BHI) broth (Oxoid) and Luria–Bertani (Oxoid) agar, respectively. For liquid growth, bacteria were grown in broth to an absorbance (A) of 0.4 for 24 h. For determination of sensitivity, bacteria were grown in liquid medium to mid-logarithmic phase at 30 °C, then split and incubated at 30 °C or 37 °C for a further 1 h. A total of 1 × 106 colony-forming units were incubated with serial dilutions of pooled human serum for either 10 or 20 min, and the proportion of bacteria surviving was determined; significant differences were examined with Student’s t-test. Total RNA was isolated using the RNAeasy Miniprep kit (Qiagen). Northern blotting was performed on 1.2% agarose, the RNA transferred to Hybond-N+ membranes, which were incubated with primary then secondary antibodies. β-galactosidase assays were performed as described previously using o-nitrophenyl-β-galactoside (Thermo Scientific Pierce) (4 mg ml⁻¹) as the substrate; reactions were stopped by the addition of NaCO3. FACS analysis was used to determine the amount of capsule has been described previously11. To determine the effect of temperature on complement sensitivity, bacteria were grown in liquid media to mid-logarithmic phase at 30 °C, then split and incubated at 30 °C or 37 °C for a further 1 h. A total of 1 × 106 colony-forming units were incubated with serial dilutions of pooled human serum for either 10 or 20 min, and the proportion of bacteria surviving was determined; significant differences were examined with Student’s t-test. Total RNA was isolated using the RNAeasy Miniprep kit (Qiagen). Northern blotting was performed on 1.2% agarose, the RNA transferred to Hybond-N+ membranes (GE Healthcare) and hybridized in Rapid-hyb buffer (GE Healthcare). In vitro transcription/translation was performed with an E. coli S30 Extract system for Linear Templates in vitro transcription/translation kit (Promega) according to the manufacturer’s instructions. RNA secondary structures were predicted using the Vienna RNA package (http://www.tbi.univie.ac.at/~ivo/RNA/).

Online Content Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Contributions E.L., E.K., A.T., Q.Z., B.G. and H.E. performed the experiments and analysed the data. R.C. and V.P. oversaw the DNA footprinting and construction of transcription factor mutants. C.M.T. provided overall direction, and wrote the manuscript with input from E.L., E.K. and R.C., and the other authors.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to C.M.T. (christoph.tang@path.ox.ac.uk).
METHODS

Bacterial strains and growth conditions. Neisseria was grown in brain heart infusion (BHI) broth (Oxoid, 37 g dissolved in 11 dH2O with 1 g soluble starch) or on BHI agar (1% w/v) supplemented with Levinthal’s base (500 ml defibrinated horse blood, autoclaved with 11 BHI broth). Solid media was inoculated from frozen stocks of bacteria stored in media with 15% glycerol at −80 °C. Cultures were then incubated for 16–18 h at 37 °C with 5% CO2. Liquid cultures were grown in 10 ml of media inoculated with 1 × 108 bacteria and grown at 37 °C with shaking (150 r.p.m.) to an absorbance of 0.6 (A600nm) of ~0.4 unless otherwise stated. N. meningitidis disease isolates belonging to serogroups B, C, W135 and Y from cc11, 32, 41/44 and 269 were described previously28.

E. coli was grown in Luria–Bertani (LB) broth (2% w/v in dH2O; Oxoid) or on LB agar (1% w/v) plates. All liquid E. coli cultures were grown in 5 ml of media inoculated from a single colony overnight at 37 °C and exposed to ECL Hyperfilm. Anti-peptide antibodies were generated against CsaA (amino acid sequences, YGTRYKTEVNTQYH of DGVTGRQSNHRMGKSI, Eurogentec), and used at a final dilution of 1:500. An anti-ReCa rabbit antibody (Bio Academica) was used at a final dilution of 1:10,000. Goat-anti-rabbit IgG HRP-conjugated antibody (Dako) was used at a final dilution of 1:10,000, whereas the anti-His-HRP conjugated monoclonal antibody (Qiagen) was used at a 1:50,000 dilution. Antibodies were used at a final concentration as follows: anti-PorB monoclonal antibody (NIBSC), 1:1,000; anti-RmpM monoclonal antibody (NIBSC), 1:1,000; anti-PilE mouse polyclonal antibody, 1:5,000; anti-fHbp mouse polyclonal antibody, 1:5,000; anti-Lst rabbit polyclonal antibody, 1:2,000.

Screen of transcription factor mutants. From the library of transcription factor mutants (Supplementary Table 1), genomic DNA from each mutant was used to transform N. meningitidis containing cssA-iacZ2 translational fusions of promoters. Colony PCR of transfomants was used to verify the mutation of genes encoding transcription factors. Once generated, the mutant library was analysed using the β-galactosidase assay.

RNA isolation and northern analysis. N. meningitidis and E. coli were grown in liquid culture to an A600nm of ~0.4 before RNA extraction. RNA was isolated using the RNAeasy MiniPrep Kit (Qiagen) following the manufacturer’s protocol. Samples was analysed by measuring at A260 nm and A532 nm. For northern blotting, 20 μg of total RNA was separated on a formaldehyde agarose gel before transfer. Hyb-bond N membranes were subsequently hybridized with 32P-labeled DNA fragments amplified with corresponding primers. Northern blots were developed and band intensities measured in a Fuji phosphorImager scanner. Primers are used in Supplementary Table 2. To amplify a DNA fragment for detection of cssA and the mutant used Csa-U and ssrA-(EC)-F with ssrA-(EC)-R.

Electrophoretic mobility shift assays. Plasmids used in electrophoretic mobility shift assays were generated by amplifying a truncated region of the intergenic region (TR, +40 to +139, primers R1F and R1R) or a full-length region of the IGR (FL, +80 to +133, primers R2F and R2R) by PCR from genomic DNA. PCR products were ligated into pGEM-T Easy (Promega), and inserted end-labelled using the Klenow fragment of DNA polymerase (New England Biolabs) and [32P]dCTP. Binding was performed in 20 mM Tris-HCl, pH 7.5, 10 mM dithiothreitol (DTT), 15% glycerol, 100 mM KCl and 0.05 mg ml−1 BSA. Dilutions of HIF and FIS were prepared in 1× reaction buffer with 1 mg ml−1 BSA. Concentrations of salmon sperm DNA and proteins are indicated. Control DNA included Tn10 and Him6.

Topo-print assay. Templates for in vitro transcription of wild-type, Δ8 and ΔA were constructed by PCR using the primers S3(TOE)-77F and S3(TOE)-new. In vitro transcription was performed using the RibolMAX Large Scale RNA production system-Sp6 and T7 kit as described by the manufacturer (Promega). In vitro transcribed RNA was ethanol precipitated, resuspended in formamide loading dye and separated on an 8% denaturing polyacrylamide gel. The RNA was visualized by ultraviolet shadowing, excised from the gel and transferred to 300 μl 2 M ammonium acetate. After overnight incubation at 14 °C, the RNA was phenol extracted followed by ethanol precipitation. Quantification was performed on a NanoPhotometer (Implen). In vitro transcribed RNA was 5′-end-labelled using the KinaseMax kit as described by the manufacturer (Ambion). Toe-printing experiments were performed in 10 μl reactions with 0.5 pmol of wild-type, Δ8 and ΔA×TOG.

The RNA were pre-incubated at 30 °C, 37 °C or 42 °C for 20 min and subsequently mixed with 0.6 pmol of 5′-end-labelled S3(TOE)-new probe in a buffer containing 60 mM NH4Cl, 10 mM Tris-acetate, pH 7.5, 10 mM DTT, 1 mM RNaseGuard and 100 mM dNTP. The mixture was incubated 2 min at 94 °C and then placed on ice for 5 min and at 30 °C, 37 °C or 42 °C for 5 min. Two different concentrations of 305 ribosomes (0.1 and 0.5 pmol) (E. coli MRE600) were added followed by 10 min incubation. The mixture was supplemented with 10 mM uncharged RNA45 (Sigma) followed by 15 min incubation after which, 2 U of AMV reverse transcriptase was added. The reaction was stopped after 30 min by the addition of 10 μl formamide loading dye. In parallel, sequencing reactions were prepared using S3(TOE)-77F and S3(TOE)-new DNA as templates. The resulting DNA was separated on an 8% denaturing polyacrylamide sequencing gel and the resulting toe-print was measured with a Fuji phosphorImager scanner.

In vitro transcription/translation. One microgram of cssA-WT-gfp (PCR amplified using CsaA-GFP-FL) and cssA-6C-R were inserted in pEGFP-N2) and prfA-gfp27 plasmids were in vitro transcribed in an E. coli S30 Extract system for Linear Templates in vitro Transcription/Translation kit (Promega) according to the manufacturer’s instructions. In brief, cssA-WT-gfp and prfA-gfp plasmids were digested using NotI restriction enzyme and purified using QIAquick PCR purification kit (Qiagen). One microgram of cssA-WT-gfp and prfA-gfp digested plasmids mixtures were incubated at 28 °C, 30 °C, 32 °C, 34 °C, 36 °C, 37 °C and 38 °C for 1 h before transferring onto ice for 5 min. Samples were acetone-precipitated, re-suspended in
1× sample buffer, and separated on a 12% polyacrylamide gel before being transferred onto a PVDF membrane using a semi-dry blotting apparatus (Biorad). Membranes were developed following the protocol of the ECL western blotting kit (Amersham), using anti-GFP (BD-living colours) as primary antibody and an HRP-conjugated anti-mouse as the secondary antibody (Dako).

**In vitro translation.** Five micrograms of RNA from *E. coli* containing either *lst* or *fhbp* (PCR amplified using fhbp-F and fhbp-R or lst(c)-F and lst(c)-R were cloned in pGEM-T) plasmids was *in vitro* translated with the *E. coli* S30 Extract system for Linear Templates *in vitro* Transcription/Translation kit (Promega) according to the manufacturer’s instructions. The RNA mixtures were incubated at 30 °C, 37 °C or 42 °C for 1 h before transferring onto ice for 5 min. Samples were acetone-precipitated, re-suspended in 1× sample buffer, and separated on a 12% polyacrylamide gel before being transferred onto a PVDF membrane using a semi-dry blotting apparatus (Biorad). Development of the membrane essentially followed the protocol of the ECL western blotting kit (Amersham), using anti-LST or anti-FHBP as the primary antibody and HRP-conjugated anti-rabbit or anti-mouse, respectively, as secondary antibody (Dako).

**Human serum sensitivity assay.** Bacterial strains were grown on BHI agar plates overnight and re-suspended in PBS. Bacteria were diluted to a final concentration of 1 × 10⁵ c.f.u. ml⁻¹ in DMEM-glutaMAX medium (Invitrogen), and incubated with different concentrations of normal human sera at 37 °C in the presence of 5% CO₂ for 1 h. Survival of bacteria in the presence of sera was determined by plating 10 μl aliquots to BHI plates and counting the number of colonies after overnight incubation. The percentage survival was calculated by comparing the number of colonies present in samples with serum to those without serum.

To compare the sensitivity of bacteria at different temperatures, *N. meningitidis* was grown in BHI broth to mid-logarithmic phase at 30 °C, then split and incubated at 30 °C or 37 °C for a further 1 h. One-million c.f.u. were incubated with serial dilutions of pooled human immune serum for either 10 or 20 min, and the proportion of bacteria surviving was determined by plating 10 μl aliquots onto BHI plates and counting the number of colonies after overnight incubation; differences were analysed with the Student’s *t*-test.

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