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Temperature triggers immune evasion by *Neisseria meningitidis*

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Abstract

Neisseria meningitidis has multiple strategies to evade complement-mediated killing, which 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 elevation in ambient temperature. We have identified three independent RNA thermosensors located in the 5′-UTRs 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 killing[1,2]. Therefore increased temperature (which occurs during inflammation) acts as a 'danger signal' for the meningococcus which enhances defence against human immune killing. Infection with viral pathogens, such as influenza, leads to inflammation in the nasopharynx with an elevated temperature and recruitment of immune effectors[3,4]. 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 meningitis[5], with peaks of disease often preceded by influenza outbreaks in temperate climates[6]. The bacterium has evolved exquisite mechanisms to evade immune responses[7], including expression of a polysaccharide capsule (containing sialic acid in serogroup B, C, Y and W strains)[8,9], sialylation of lipopolysaccharide (LPS), and recruitment of the human complement regulator factor H, via high affinity interactions with bacterial factor H binding protein[1-2]. 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 following insertion of the mobile element IS*1301* into the 134 bp intergenic region (IGR) between the *css* (encoding capsule biosynthesis) and *ctr* (capsule export) operons in the capsule biosynthesis locus (*cps*)[10,11]. To identify other changes modulating serum resistance, we subjected the *N. meningitidis* strain S3[11] to serial passage

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Author contributions

EL, EK, AT, QZ, BG and HE performed the experiments and analysed the data. RC and VP oversaw the DNA footprinting and construction of transcription factor mutants. CMT provided overall direction, and wrote the manuscript with input from EL, EK and RC, and the other authors.

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in 6% human serum; within six rounds, S3 became as resistant to complement-mediated killing as a strain (R3) with IS*1301* in the IGR (Supplementary Fig. 1). We further characterised six passaged strains (selected serum resistant, SSR 1-6) that were more resistant to complement than S3 (Fig. 1a). Resistance did not result from insertion of IS*1301*; instead five strains (all except SSR2) have lost a single copy of a duplicated 8 nt. sequence (TATACTTA) located 15 nt. upstream of the CssA start codon in the 5′-untranslated region (5′-UTR) of *css* mRNA (Fig. 1b-c, Supplementary Fig. 2) and have elevated levels of CssA, which catalyses the first step in capsule biosynthesis[12]. Comparison of S3 with SSR, and isogenic strains (both containing an antibiotic resistance cassette downstream of the *css* operon) with one (Δ8) or two copies (wild-type, WT) of the 8 bp sequence demonstrates that loss of 8 bp causes elevated 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 antibodies[13].

To define how the 8 bp sequence contributes to capsule expression, we performed DNA footprinting of the IGR with IHF and FIS. Despite potential recognition sites for these proteins[10,14], there was no difference in IHF or FIS binding to the WT 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 Fig. 4 and 5), demonstrating that Δ8 influences the capsule biosynthesis operon. Furthermore, significantly elevated Css reporter activity with the Δ8 IGR was evident in 38 transcription factor mutants (Supplementary Table 1, Supplementary Fig. 5), while northern blot analysis demonstrates 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-transcriptionally.

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 thermosensor[15]. In RNA thermosensors, the transcript assumes a hairpin structure at lower temperatures that occludes the RBS, and stalls protein translation; higher temperatures destabilise 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). In 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 *E. coli* containing CssA and the WT 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 elevation 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 (nt.) changes into the 5′-UTR predicted to alter the stability of the thermosensor, including substitutions at the same position ($+92_{U/C}$ or $+92_{U/G}$, 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 toeprinting 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 WT 5′-UTR, with differences most marked at 30°C (Supplementary Fig. 6). Finally we found that thermoregulation of CssA is 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 *N. meningitidis* across a range of strains.

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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[15-18]. As the meningococcus is an obligate commensal residing in the nasopharynx, we compared the dynamic response of the canonical PrfA *Listeria* thermosensor with the meningococcal Css thermosensor (which directs a single pathway). The *prfA* and *css* 5′-UTRs and their promoters were fused to GFP in pEGFP-N2, and *in vitro* transcription/translation was performed at different temperatures (Fig. 3a). Protein synthesis regulated by the *prfA* thermosensor was barely detected up to 36°C, but markedly increased at higher temperatures. In contrast to this on:off switching, the *css* thermosensor displayed a gradual increase over physiologically relevant temperatures (acting like a rheostat). The difference can be explained by the relatively high GC content and distribution of GC bonds flanking the RBS in the *prfA* 5′-UTR (Fig. 3b); in the bacteria, the *Listeria* thermosensor mediating a marked temperature shift as the bacterium migrates from the external environment into its host, while *N. meningitidis* is in virtual constant contact with its host and therefore be less exposed to large temperature wide temperature fluctuations.

Next we determined the prevalence of IGR polymorphisms in 265 meningococcal disease isolates. The WT sequence (two copies of the 8 nt. sequence) was most frequently found (201 of 265, 75.8%); of note, no polymorphism was detected in the 5′-UTR with two copies of the 8 nt. Strains with a Δ8 accounted for the remainder (64/265, 24.2%, Fig. 3c), although this deletion was accompanied by two substitutions (TAT<u>G</u>C<u>C</u>TAT; altered bases underlined, $\Delta 8_{AT/GC}$) in the majority of instances (50/64 isolates, 78.1%). Only a few strains (8/64, 12.5%) had the Δ8 sequence with no changes, and the single, TAT<u>G</u>CTTAT polymorphism ($\Delta 8_{A/G}$) was present in the other strains (6 of 64, 9.4%), but TATAC<u>C</u>TAT ($\Delta 8_{T/C}$) was never detected. We analysed the effect of these polymorphisms in *E. coli* plasmid reporters (Fig. 3d), and found that whereas $\Delta 8_{A/G}$ partially restores CssA thermal regulation following deletion of 8 nt., $\Delta 8_{AT/GC}$ re-establishes thermosensing (consistent with its predicted structure and RNA toe-printing, Supplementary Fig. 6 and 7). In contrast, $\Delta 8_{T/C}$ (which was never found) leads to markedly elevated CssA 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 WT sequence and prevalence of compensatory polymorphisms emphasise 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 LPS sialylation[19]) 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 in *E. coli* plasmids harbouring 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), while 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°C then incubated at 30°C or 37°C for one hour. Bacteria that had been equilibrated at the elevated

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temperature expressed more CssA, fHbp, and Lst (Supplementary Fig. 10) and were significantly more resistant against complement than those left at 30°C (Fig. 4b), demonstrating that thermal regulation of immune defence mechanisms has a marked impact on bacterial survival in the presence of human complement.

An elevation 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 airway[3], a rise in core temperature, and elaborates neuraminidase that could damage the meningococcal capsule[20]. By sensing local inflammation, thermal regulation of immune defence by the meningococcus would allow adaption to changes in the nasopharynx during infection with influenza[21,4] and other respiratory pathogens; microbes unable to sense and protect themselves against inflammatory responses will face elimination from this habitat (Fig. 4b). Furthermore, thermoregulation by the meningococcus would provide an advantage to bacteria entering the bloodstream, which is at higher temperatures than the nasopharynx[22]. Therefore traits that are beneficial for bacterial colonisation during coinfection and inflammation (such as thermoregulation of immune defence) will, by unfortunate coincidence, promote the virulence of otherwise commensal microbes.

Materials and Methods

Bacterial Strains and Growth Conditions

Neisseria was grown in Brain Heart Infusion broth (BHI, Oxoid, 37 g dissolved in 1 L dH_2O with 1 g soluble starch) or on BHI agar (1% w/v) supplemented with Levinthal's base (500 ml defibrinated horse blood, autoclaved with 1 L 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 hours at 37°C with 5% CO_2. Liquid cultures were grown in 10 ml of media inoculated with 10^9 bacteria and grown at 37°C with shaking (150 r.p.m.) to an optical density (O.D.) measured at 600 nm (A_{600}) 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 previously [1]

E. coli was grown in Luria-Bertani (LB) broth (2% w/v in dH_2O, Oxoid, UK) 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 with shaking (250 r.p.m.). Overnight grown bacteria were diluted 1/100 in media and grown to an A_{600} of ~0.4 unless otherwise stated. To construct isogenic strains, isolates were tagged with a kanamycin cassette in *oatC* as previously described [2]. Primers NG1095-for and NG1125-rev were used to amplify an 8 kb region across the IGR and *oatC* using genomic DNA from SSR5 as DNA template; all primers are shown in Supplementary Table 2. The PCR product was sequenced, purified and transformed into S3. Eight transformants with the deletion and four without the deletion were analysed to confirm the resistance phenotype. The *porA* gene was amplified and sequenced with NG1945-for and NG1946-rev.

Plasmid DNA from *E. coli* was isolated from overnight cultures grown in LB broth using the GenElute™ Plasmid Kit (Sigma-Aldrich). Genomic DNA from *N. meningitidis* was purified as previously, and PCRs were performed using a PTC-225 Tetrad PCR machine (MJ Research). DNA was purified using a QIAquick gel extraction kit (Qiagen, UK) and DNA digestion was carried out using enzymes supplied by New England Biolabs, Ligation reactions contained no more than 100 ng of purified vector and insert DNA (usually in a 1:3 ratio) and Quick-stick ligase (Bioline, UK). *N. meningitidis* was transformed by placing 10

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μl of a bacterial suspension on solid media, and allowing it to dry. Plasmid or genomic DNA was added to the bacteria, and the plates were incubated for 4 hours at 37°C before streaking on selective media.

N. meningitidis reporter strains

Reporter constructs for *N. meningitidis* were initially generated in pUC19 in *E. coli* as follows. The erythromycin resistance cassette was amplified from pYHS1882 using NG1304 and NG1305. The resulting product and pUC19 were digested with *Aat*II and *Eco*01091 and ligated together. The resulting construct was digested with *Bsa*I and *Aat*II and ligated with a region of the *cssA* ORF amplified using primers NG1306 and NG1307 and digested with the same enzymes. Promoters of interest were then inserted upstream of the erythromycin resistance cassette following *Hind*III and *Afl*III digestion. The promoter regions were amplified from strains with primers NG1324 and NG1419. A truncated *ctrA* with no intergenic region (IGR) was amplified with NG1419 and NG1420, and integrated into the pUC19. Next the *lacZα* fragment was removed from the vector by digestion with *Hind*III and *Sfo*I, and replaced with full length *lacZ* amplified from pRS415 using NG1480 and NG1484. The resulting fusions contained the *cssA* start codon fused to *lacZ* at codon 10. Constructs were verified by restriction mapping and sequence analysis before transformation of *N. meningitidis*. Integration of the vector by double crossover leads to a single chromosomal copy of the *lacZ* reporter in the capsule biosynthesis locus. Template genomic DNA was used with a WT or Δ8 IGR as appropriate.

Whole cell lysates, *β*-galactosidase assays and FACS analysis

N. meningitidis strains were harvested after overnight growth on BHI agar plates and re-suspended in PBS. The concentration of the bacterial suspension was determined by measuring the A_{260} of a mixture containing 20 μl of the bacterial suspension and 980 μl of lysis buffer (0.1 M NaOH, 1% SDS) using a UVVIS spectrophotometer (Shimadzu UK). The bacterial suspension was adjusted to 10×10^9 CFU/ml, mixed with an equal volume of 2× SDS-PAGE loading buffer (100 mM Tris-HCl pH 6.8, 20 μM *β*-mercaptoethanol, 4% SDS, 0.2% bromophenol blue, 20% glycerol), and boiled for 10 minutes. The samples were centrifuged at 16,000 ×*g* for 20 seconds, and 10 or 20μl of samples was analysed by SDS-PAGE. *β*-galactosidase assays were performed as described previously [3] using *o*-nitrophenyl-*β*-galactoside (ONPG) (4 mg/ml) as the substrate; reactions were stopped by the addition of 1 M $NaCO_3$. FACS analysis to determine the amount of capsule has been described previously[2]. Results were calculated as mean fluorescence index (MFI), the geometric mean multiplied by the percentage of positive cells.

SDS-PAGE and Western blotting

Proteins were separated on polyacrylamide gels alongside Precision Plus All Blue markers (Biorad, USA) and stained using Coomassie blue stain for 10 minutes. Proteins were transferred to immobolin P polyvinylidine fluoride (PVDF) membranes (Millipore, USA) using the wet transfer system (Biorad, USA). For Western blot analysis, membranes were washed three times in 0.05% (w/v) dry milk/PBS with 0.05% (v/v) Tween-20 for 10 minutes, and then incubated with the primary antibody for two hours. Membranes were washed again three times and incubated for a further hour with a secondary, HRP-conjugated antibody. Binding was detected with an ECL Western Blotting Detection kit (Amersham, USA) and exposed to ECL Hyperfilm. Anti-peptide antibodies were generated against CssA (amino acid sequences, YGRTYKEVTRENYQH or DVGTRQSNRHMGKSI, Eurogentec), and used at a final dilution of 1:500. An α-RecA rabbit antibody (Bio Academia, France) was used at a final dilution of 1:10,000. Goat α-rabbit IgG HRP-conjugated antibody (Dako, UK) was used at a final dilution of 1;10,000, while the α-His-HRP conjugated mAb (Qiagen, UK) was used at a 1:50,000 dilution. Antibodies were used

at a final concentration as follows: α-PorB mAb (NIBSC), 1:1000; α-RmpM mouse mAb (NIBSC), 1:1000; α-PilE mouse pAb, 1:5000; α-fHbp mouse pAb, 1:5000; α-Lst rabbit pAb, 1:20000.

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 *css*::*lacZ* translational fusions of promoters. Colony PCR of transformants 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 O.D. A_{600} of ~0.4 prior to RNA extraction. RNA was isolated using the RNAeasy Miniprep Kit (Qiagen, UK) following the manufacturer's protocol. Samples was analysed by measuring the A_{260} and A_{280}. For northern blotting, 20μg of total RNA was separated on a formaldehyde agarose gel prior to transfer. Hybond-N membranes were subsequently hybridised with $^{32}P\gamma$-labeled DNA fragments amplified with corresponding primers. Northern blots were developed and band intensities measured in a Fuji phosphorImager scanner. Primers used are listed in Table 2. To amplify a DNA fragment for detection of *sscA* and tmRNA, we used CssA-U with CssA-U and ssrA-(EC)-F with ssrA-(EC)-R.

Electrophoretic Mobility Shift Assays

Plasmids used in Electrophoretic Mobility Shift Assays (EMSA) 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 +132, primers R2F and R2R) by PCR from genomic DNA. PCR products were ligated into pGEM-T Easy (Promega), and inserts end labelled using the Klenow fragment of DNA polymerase (New England Biolabs, UK) and [α-^{32}P] dCTP. Binding was performed in 20 mM Tris-HCl, pH7.5, 10 mM DTT, 15% glycerol, 100 mM KCl and 0.05 mg/ml BSA. Dilutions of IHF and FIS were prepared in 1 × reaction buffer with 1 mg/ml BSA. Concentrations of salmon sperm DNA and proteins are indicated. Control DNA included Tn10 and Himar [4].

Toe-print assay

Templates for *in vitro* transcription of WT, Δ8bp, and $\Delta 8bp_{AT/GC}$ were constructed by PCR using the primers S3(TOE)T7F and S3(TOE)-new. *In vitro* transcription was performed using the RiboMAX™ Large Scale RNA production sytems-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 UV shadowing, excised from the gel and transferred to 300 μl 2M NH_4Acetate. 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 WT, Δ8bp, and $\Delta 8bp_{AT/GC}$. The RNA were pre-incubated at either 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 60mM NH_4Cl, 10mM Tris-acetate [pH 7.5], 10mM DTT, 1 ml RNAguard and 100 mM dNTP. The mixture was incubated 2 min at 94°C and then placed on ice for 5 min and either at 30°C, 37°C or 42°C for 5 min. Two different concentrations of 30S 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

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tRNA^{fmet} (Sigma) followed by 15 min incubation after which, 2U 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)T7F 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 CssA-GFP-F(L) and CssA-6C-R were inserted in pEGFP-N2) and prfA-gfp[5] 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 *Not*I 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 hour 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 microgram 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 were *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 either 30°C, 37°C or 42°C for 1 hour 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 1×10^5 CFU/ml in DMEM-glutaMAX™ medium (Invitrogen, UK), and incubated with different concentrations of normal human sera (NHS) at 37 °C in presence of 5% CO_2 for 1 hour. 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 percent 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 hour. 10^6 CFU were incubated with serial dilutions of pooled human immune serum for either 10 or 20 mins, 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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Supplementary Material

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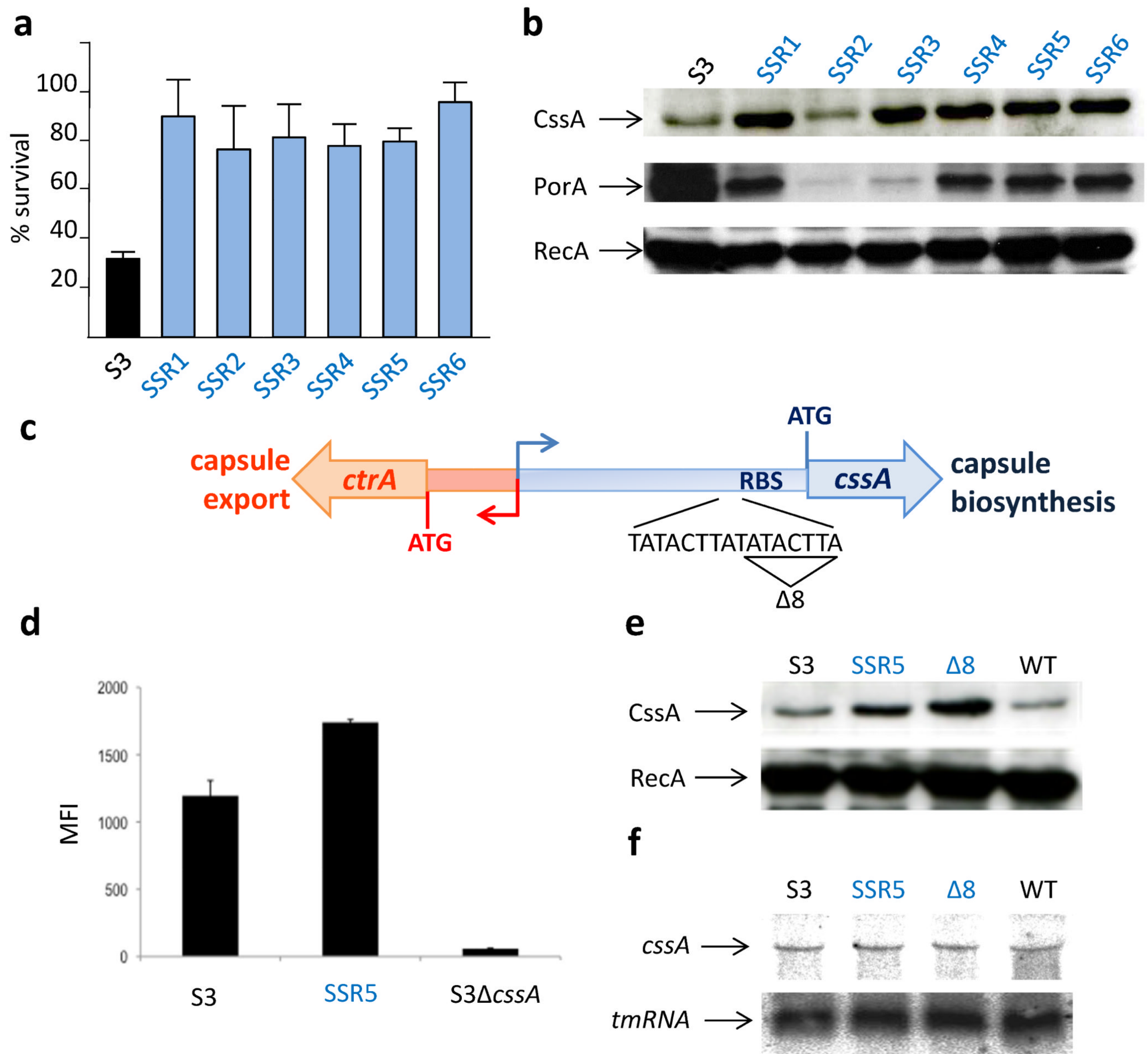

Fig. 1. Loss of an 8 bp increases capsule expression and complement resistance

Selected serum resistant strains (SSR1-6) 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 (arrows) 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 elevated *cssA* mRNA. Findings were confirmed in three biological replicates. Error bars show S.D. of experiments performed in triplicate.

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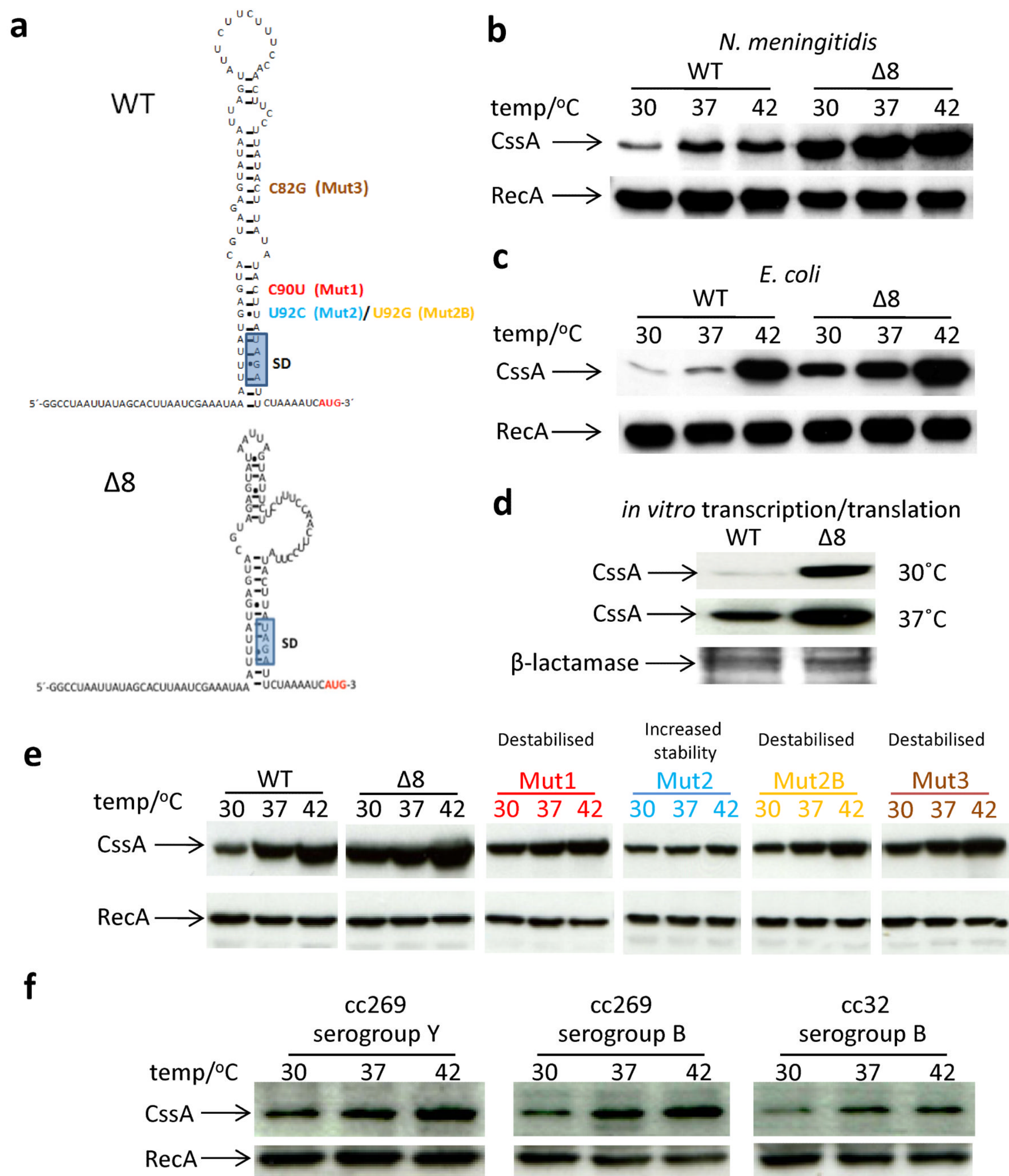

Fig. 2. Capsule gene expression is governed by an RNA thermosensor

(a) Predicted secondary structure of the *css* 5′-UTR with two (WT) or one copy of the 8 nt. sequence (Δ8); the ribosome binding site is indicated. (b) Western blot analysis of CssA levels in isogenic *N. meningitidis* strains with a wild-type IGR (WT) or containing a single copy of the 8 bp sequence (Δ8) grown at different temperatures (indicated above each lane). (c) Thermoregulation of CssA expression is detected in *E. coli* by western blot analysis, and (d) by *in vitro* transcription/translation assays with purified *E. coli* RNA polymerase; *β*-lactamase included as a control. (e) Mutational analysis of the 5′-UTR. *E. coli* expressing CssA with modifications (shown in panel a) grown at temperatures indicated; the predicted effects shown. (f) Thermoregulation of CssA is evident in *N. meningitidis* across different

capsular serogroups and lineages (cc, clonal complex). Findings were confirmed with three biological replicates.

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Fig. 3. Thermosensing of immune evasion mechanisms

(a) *In vitro* transcription/translation assays and (b) predicted structure of the Css and PrfA thermosensors modulating GFP expression in *E. coli* at a range of temperatures. (c) Sequence analysis of the IGR in different hypervirulent lineages; clonal complexes (cc) shown. Loss of a copy of the 8 bp sequence (Δ 8) is associated with a 2 bp substitution in the remaining single 8 bp sequence (*i.e.* $\Delta 8_{AT/GC}$), which is never detected in a strain with two copies of the 8 bp sequence. (d) Effect of the polymorphisms examined in *E. coli*; the most abundant polymorphism $\Delta 8_{AT/GC}$ restores thermosensing to wild-type levels in the presence of a single 8 bp sequence, whereas $\Delta 8_{A/G}$ substitution partially restore thermosensing; $\Delta 8_{T/C}$ leads to higher CssA levels than Δ8. (e) Western blot analysis demonstrates that levels of

proteins mediating immune evasion, fHbp and Lst, increase in *N. meningitidis* with an elevation in temperature, (f) whereas other proteins (indicated) are unaffected. Blots are representative of experiments performed on at least three occasions.

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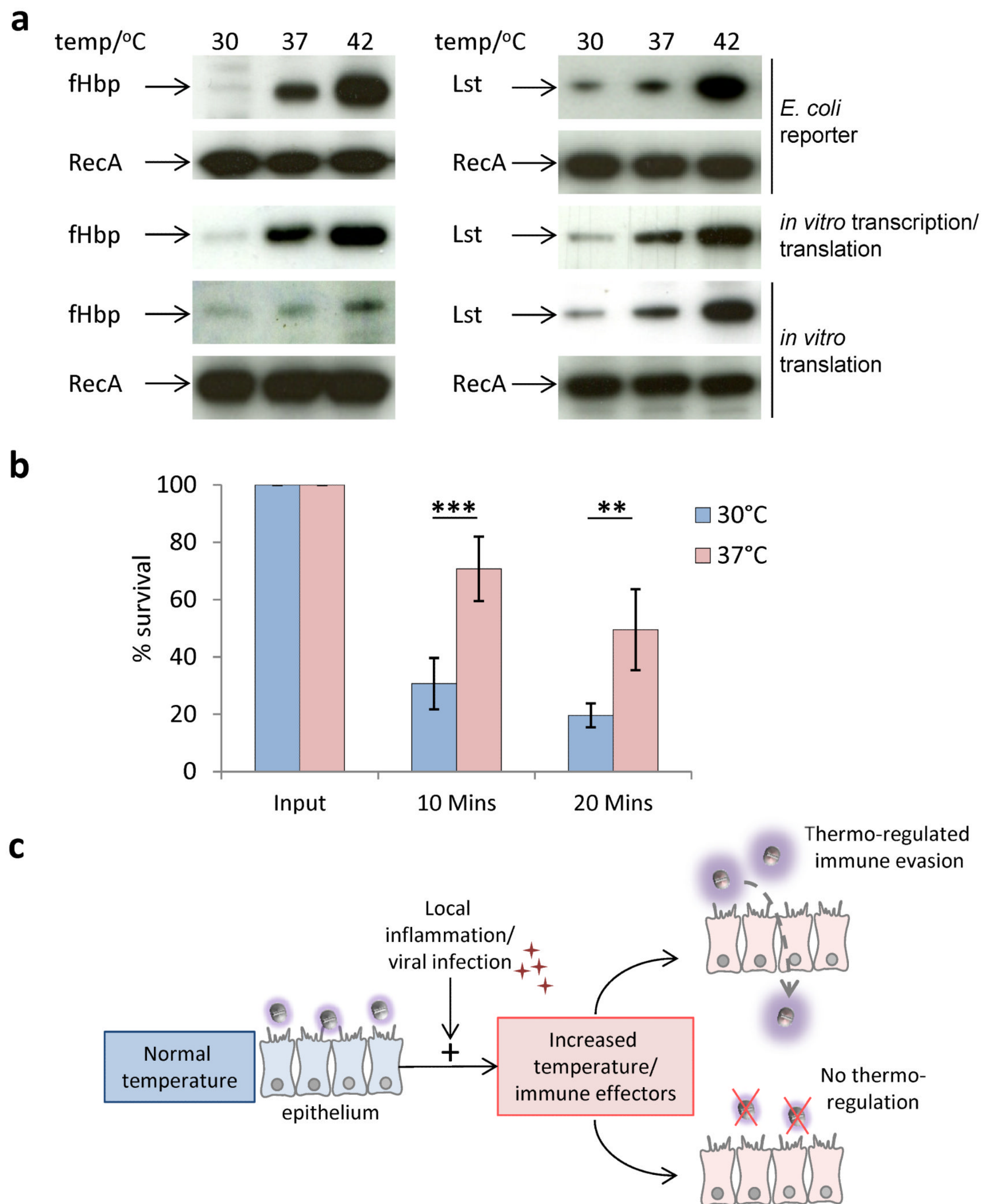

Fig. 4. Temperature influences complement escape by *N. meningitidis*

(a) Thermoregulation of fHbp and Lst expression in i) *E. coli* reporters detected by Western blot analysis; RecA, loading control, ii) *in vitro* transcription/translation assays, and iii) *in vitro* translation assays of fHbp and Lst; RecA, expression control. Blots are representative of experiments performed on at least three occasions. (b) Bacteria incubated for 1 hr at 37°C are more resistant to complement-mediated killing than those at 30°C: the assay was performed on four separate occasions and error bars represent the SEM; ** and ***, $p<0.01$ and <0.001 (Students T test). (c) Inflammation at the epithelial surface in the nasopharynx (in response to viral co-infection) would increase local 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.

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