SUPPLEMENTARY FIGURES

Fig. supp. 1

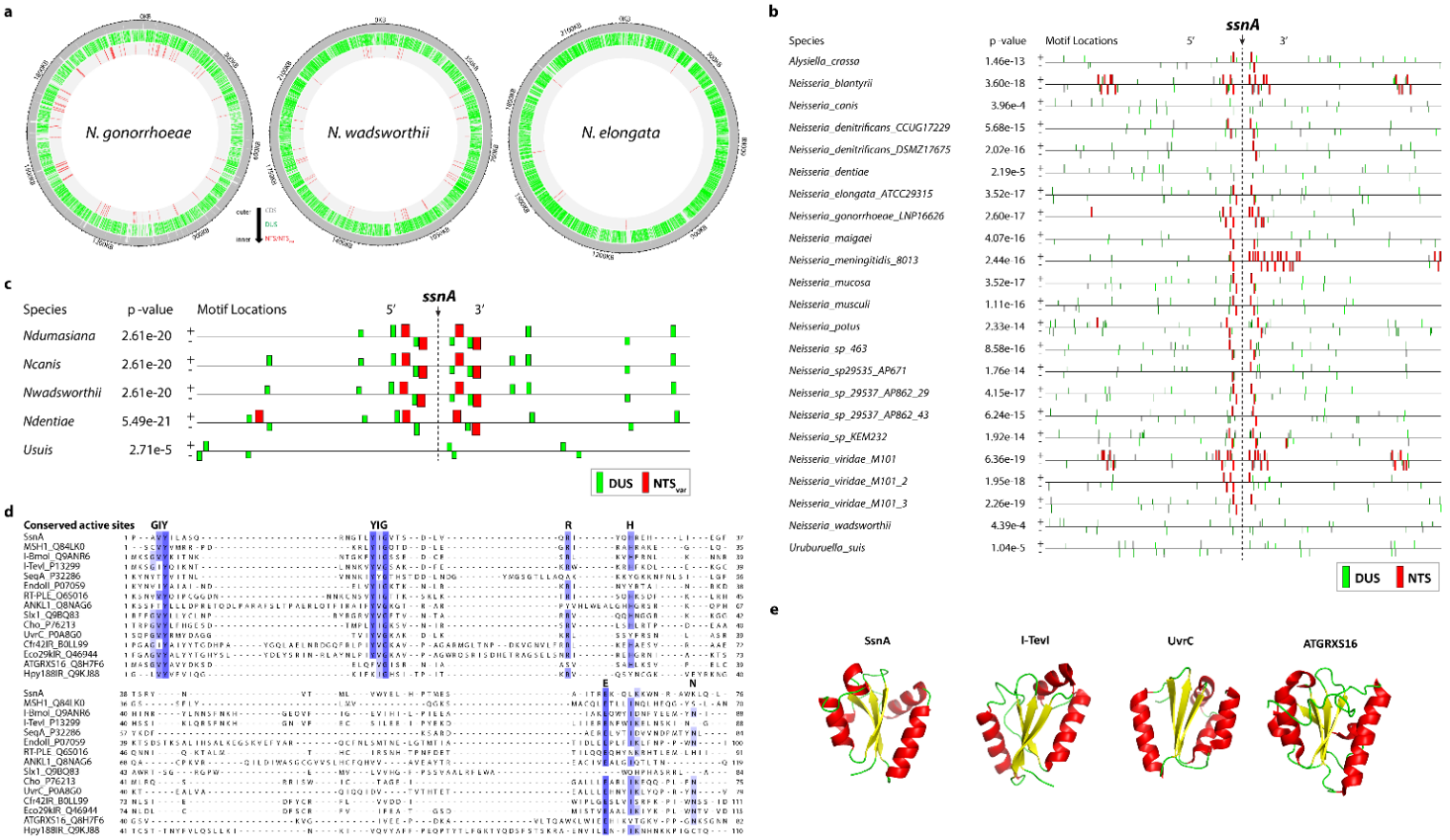


Figure S1

a, Whole-genome representation of CDS, DUS, and NTS repeats in selected *Neisseria* species, generated with shinyCircos-V2.0. For *N. wadsworthii*, NTS_{var} are identified. b, Graphical representation of the 10 kb genomic regions flanking *ssnA* in *Neisseria* species. The two most frequent repeats were automatically plotted using MEME. c, as in (b), using only the genomes where no NTS were detected, showing the identification of the NTS_{var} motifs. d, Amino acid sequence alignment of the GIY-YIG domains from SsnA and representative proteins of known GIY-YIG families e, 3D structures of *N. meningitidis* SsnA and well-characterized GIY-YIG proteins. The SsnA structure model was inferred using the Alphafold server.

Figure Supp. 2

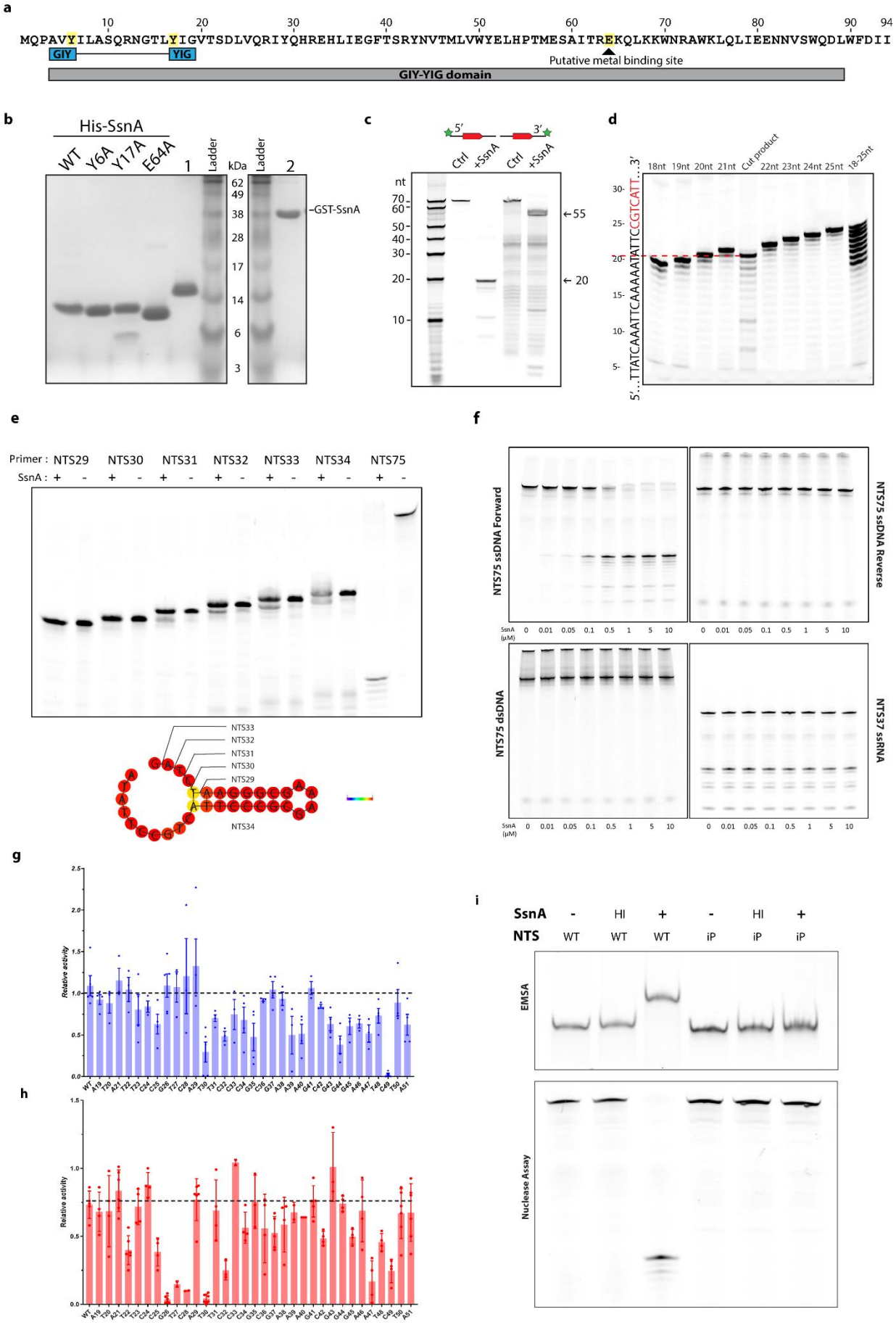
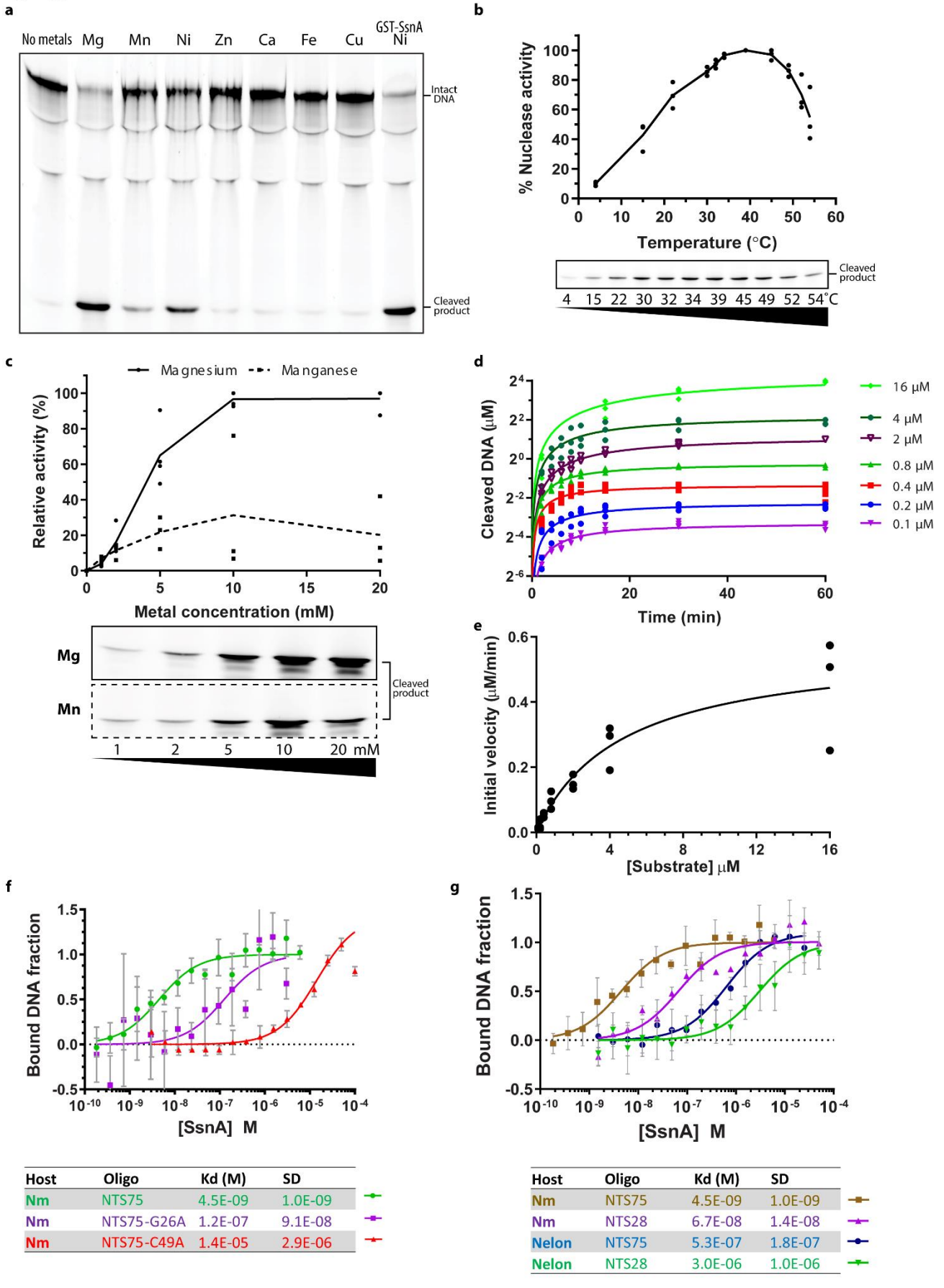


Figure S2

a, SsnA protein sequence and architecture as predicted by the CDD database. Mutated amino acids are highlighted. b, SDS-PAGE of recombinant SsnA proteins after column affinity purification. The WT and mutated (Y6A, Y17A, E64A) proteins come from *Neisseria meningitidis* 8013 2C4.3. 1- His-SsnA from *N. elongata*, 2- GST-SsnA from *N. meningitidis*. c, Nuclease activity of His-SsnA WT on a 75nt ssDNA oligo containing the NTS, labelled at either the 5' or 3'-end (5'-NTS75, NTS75-3'). d, SsnA cutting site determination. The cleaved product from NTS75 ssDNA was resolved by denaturing PAGE along with 18-25 nt sequences. e, Nuclease assay to determine the minimum sequence necessary around the NTS for cleavage. The assay was performed under standard conditions (described previously) using decreasing length primers all starting in 5' at 2 nucleotides from the cleavage site. A schematic representation of the different sequences tested is presented under the gel. f, Nuclease activity assay with different concentrations of SsnA on four substrates containing the NTS: ssDNA forward, ssDNA reverse, dsDNA, and RNA. The assays were performed under the conditions described previously, with substrate concentrations set at 0.8 µM. g, Relative binding activity of His-SsnA WT to 75 nt ssDNA sequences carrying single-nucleotide substitutions (NTS75mut), measured from gel-shift assays. Means+SEM from at least three independent experiments are shown. h**,** Relative cleavage activity of His-SsnA WT against the same sequences as in d, measured from nuclease assays. Means ±SEM from at least three independent experiments are shown. For g, and h, the n number of each column is available in Source data. i, EMSA and nuclease assays illustrating the role of the palindromic sequence in the recognition of NTS by the SsnA protein of *N. meningitidis*. The WT sequence corresponds to NTS75, and the iP represents the inversion of the palindrome sequence in NTS, altering the sequence without modifying the structure and GC percentage. HI stands for Heat Inactivated. All experiments were reproduced at least three times with similar results.

Fig. Supp. 3



Host	Oligo	Kd (M)	SD
Nm	NTS75	4.5E-09	1.0E-09
Nm	NTS75-G26A	1.2E-07	9.1E-08
Nm	NTS75-C49A	1.4E-05	2.9E-06

Host	Oligo	Kd (M)	SD
Nm	NTS75	4.5E-09	1.0E-09
Nm	NTS28	6.7E-08	1.4E-08
Nelon	NTS75	5.3E-07	1.8E-07
Nelon	NTS28	3.0E-06	1.0E-06

Figure S3

a, Metal cofactor dependency for the nuclease activity of SsnA on oligo NTS100. 10mM of each metal was added to a metal-free reaction buffer. Mn manganese, Mg magnesium, Ni nickel, Cu copper, Fe iron, Ca calcium. Unless otherwise indicated, His-tagged purified SsnA was used. b, Nuclease activity of His-SsnA on oligo NTS100 at different temperatures. Means from three independent experiments are shown (n=3 at each temperature. c, Nuclease activity of His-SsnA on oligo NTS100 in presence of varying concentrations of magnesium (Mg) and manganese (Mn). The line represents the average of independent experiments. (n=4 for Mg, n=3 for Mn). d, Cleavage kinetics of His-SsnA on NTS100 oligo. Means from at least three independent experiments are shown, fitted to a hyperbola (for 0.1/0.2/0.4μM n=4, for other concentration n= 3). e, Michaelis-Menten plot of the initial enzyme velocities obtained in d. Individual replicates are shown, fitted to a hyperbola. Km = 4.68±1.45 μM. f, Binding kinetics of SsnA from *N. meningitidis* to the indicated Cy-5 labelled oligos, where NTS75-G26A and NTS75-C49A contain single-nucleotide substitutions in the NTS repeat. Means±SEM from at least three independent experiments are shown. g, Binding kinetics of SsnA from *N. meningitidis* or *N. elongata* to the indicated Cy-5 labelled oligos, where NTS28 is a truncated version of NTS75 that contains a full NTS copy with no flanking sequences. Means±SEM from at least three independent experiments are shown. The exact n number for each point of each curve is available in the Source Data file.

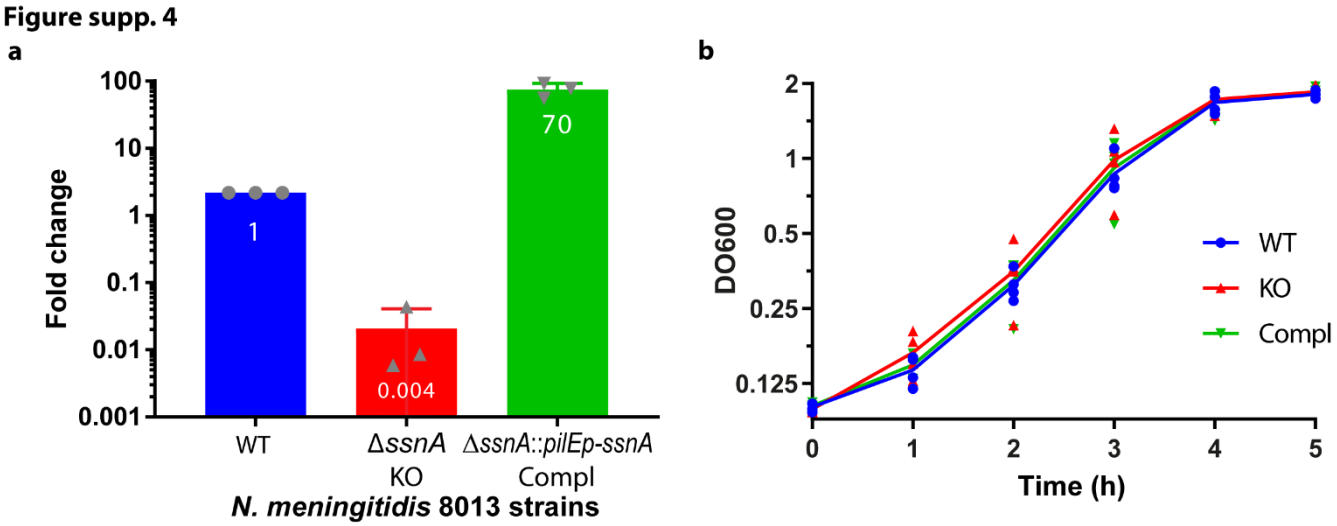


Figure S4

a, RT-qPCR of *ssnA* from the *N. meningitidis* 8013 2C4.3 strains. The complementation strain uses a strong promoter resulting in overexpression of *ssnA*. Means+SD of three independent experiments are reported (n= 3 for each strain). b, Growth curves of the SsnA mutants under optimal conditions. The line represents the average of the biological replicates (n=4 for each strain at each time point).

Fig. supp. 5

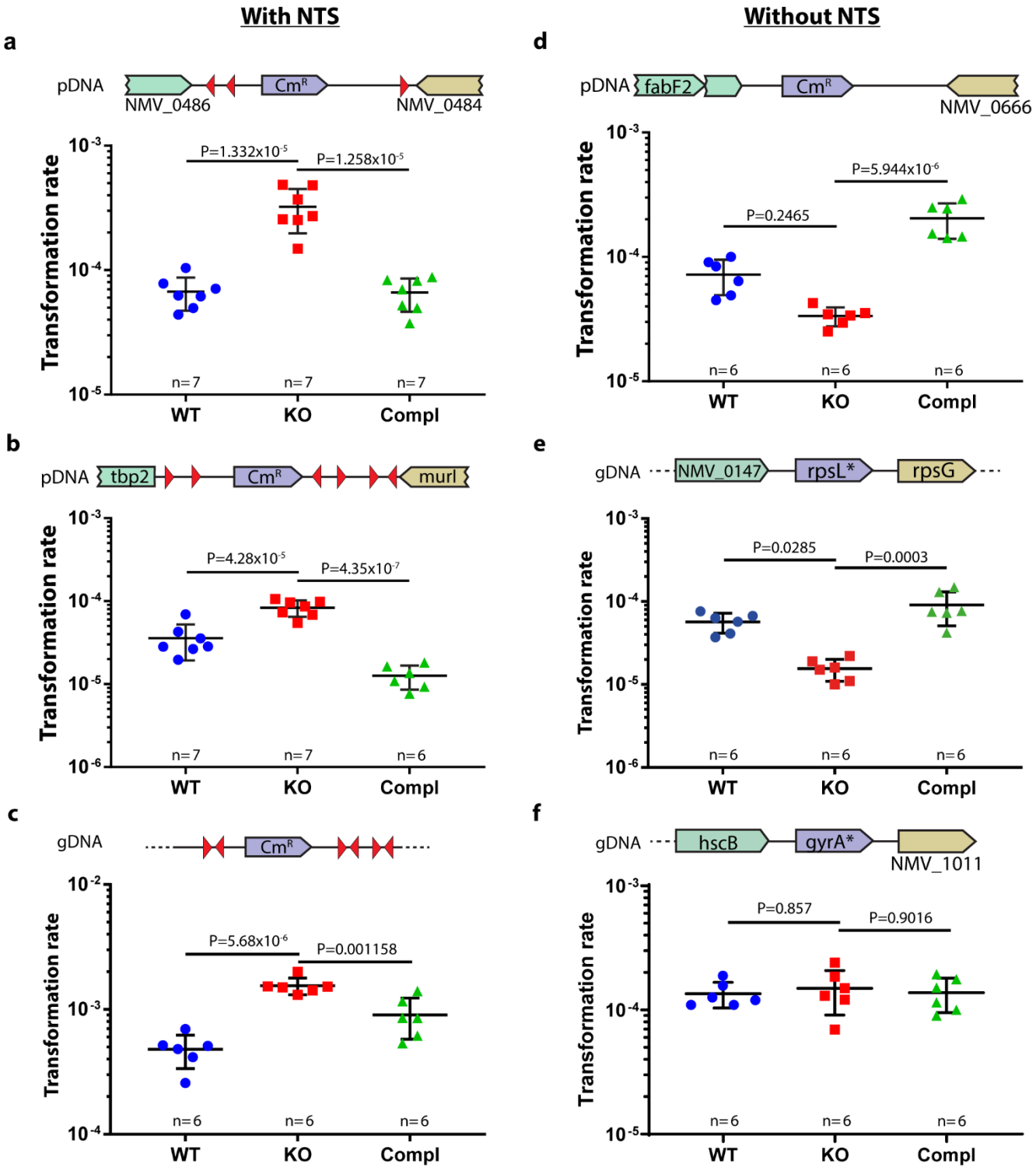


Figure S5

Transformation rates of *N. meningitidis* strains using DNA substrates a,b,c, containing NTS repeats such as plasmids (a, b) and genomic DNA (c), or d,e,f, without NTS repeats in the recombination regions. Each substrate integrates in a different genomic region. For all assays, individual transformation reactions from at least three independent experiments are shown with the mean±SD. one-way ANOVA with post-hoc Tukey's multiple comparisons test.

SUPLEMENTARY METHODS

Bacterial strains and culture conditions

Bacterial strains used in this study are listed in Supplementary Data 6.

Escherichia coli strains were cultured at 37°C on Luria-Bertani (LB) media supplemented when required with 100 µg/ml ampicillin (Amp), 50 µg/ml kanamycin (Km), 25 µg/ml chloramphenicol (Cm), or 300 µg/ml erythromycin (Ery). Cloning procedures were performed using the DH5α strain and protein expression was performed using the BL21(DE3) strain.

Neisseria strains were grown at 37°C with 5% CO_2 on GC agar base (GCB, Oxoid) enriched with Kellogg's supplements[1]. When required, GCB plates were supplemented with 5 µg/ml chloramphenicol (Cm), 200 µg/ml streptomycin (Sm), 100 µg/ml kanamycin (Km), 3 µg/ml erythromycin (Ery).

N. meningitidis 8013 2C4.3 is a piliated and capsulated variant of the serogroup C isolate 8013[2]. *N. meningitidis* LNP24198lux and LNP20553 were gifted by Muhamed-Kheir Taha from the Centre National de Reference des Meningocoques (Institut Pasteur, Paris, France). Both strains are of serogroup C and belong to the hyperinvasive ST-11 clonal complex[3,4].

DNA manipulations and mutant strain generation

Plasmids used to generate bacterial strains and their construction are listed in Supplementary Data 7. Standard procedures were performed following the suppliers' recommendations. Restriction enzymes and T4 DNA ligase were ordered from New England Biolabs (NEB), DNA extraction and purification kits were obtained from Qiagen, and Sigma provided the oligos. Phusion polymerase (NEB) was used for PCR of fragments to be cloned. Sequence verifications were done by Sanger sequencing at Génome Québec. To generate the different *Nm* strains used in transformation assays, the protocol for *Neisseria* markerless modifications described in [5] was followed. To generate *ssnA* knockout (KO) strains, pKOSsnA::ery was transformed in *Nm*. The KO strains were then transformed with pOESsnA::km for complementation of *ssnA*, where the gene was reinserted elsewhere in the genome under the strong *pilE* promoter. Due to antibiotic resistance limitations, complementation of *ssnA* in *Nm* LNP24198lux and in *Nm* 8013 $rpsL^{K43R}$ yebNOut::CmSTOP 5M3::Km could not be done. Each mutant strain was verified by PCR and/or Sanger sequencing (Genome Quebec).

N. meningitidis growth curves and SsnA qPCR

Growth curves were performed in liquid GC base media supplemented with Kellogg's supplements[1]. A starting OD_{600} of 0.1 was used, and cultures were incubated at 37°C, 300 rpm without CO_2 supplementation. Optical density was measured every hour using SmartSpec Plus spectrophotometer (Bio-Rad).

RT-qPCR to verify *ssnA* expression in *Nm* 8013 mutant strains was done in biological triplicates using standard procedures with the RevertAid H Minus Transcriptase (Thermo) and PowerUp SYBR Green (Thermo) in a StepOne Plus thermocycler (Applied Biosystems). *gyrA* was used as the reference gene with primers gyrA_F/R, while *ssnA* was detected using primers SsnA_F/R (Supplementary Data 8).

Neisseriaceae phylogeny

Genome annotations for the *Neisseriaceae* genome database were retrieved from the NCBI RefSeq database, and where unavailable, genomes were annotated with Prokka. A core genome nucleotide alignment was created from these annotated genomes with Roary v3.13.0 using MAFFT (Roary options options -e --mafft. Criteria for inclusion of a gene in the core-genome encompassed a minimum of 50% identity, and presence in 80% of the isolates, (-i 50 -cd 80 roary options). This resulted in a core genome alignment of 895 genes within *Neisseriaceae* which was used for subsequent. The model of nucleotide evolution which best fit these data was estimated by ModelFinder. The best-fit model according to the Bayesian Information Criterion (BIC) was GTR+F+. The subtree representing the *Neisseria* genus was extracted and rooted with *Eikenella corrodens*.

Functional annotation of *Nm* genes flanked by NTS motifs

For each protein-coding gene in the *Nm* 8013 proteome (as annotated in GCA_000026965.1), the genomic regions 5kb up and downstream from the corresponding gene were extracted using an in-house script utilizing tools from the samtools (v1.17), bedtools (v2.30.0), and exonerate (v2.4.0) programs[10-12]. Then, fuzznuc was used to search these regions for NTS repeats (in both strands, with one permitted mismatch) in order to identify all proteins in the *Nm* 8013 proteome for which at least one NTS motif was found in both the 5' and 3' flanking regions of their corresponding genes. This set of proteins was then functionally annotated based on the Clusters of Orthologous Genes (COG) database using eggNOG mapper (options --evalue 0.001 --score 60 --pident 40 --query_cover 20 --subject_cover 20)[13]. The predicted localization of each protein was then estimated using PSORTb v3.03[14].

Prediction and Comparison of Structures

The structure of the SsnA protein was predicted using the Alphafold 3 server[15] based on the protein sequence of NMV_0044. Structural comparisons were performed using the TM-Align tool[16] (Supplementary data 10).

Cloning, expression and purification of SsnA

Primers used in this study are listed in Supplementary Data 8. The *NMV_0044* (*ssnA*) gene from *N. meningitidis* 8013 2C4.3 was amplified by PCR then cloned into pET15-MHL using NdeI and XhoI sites. The Y6A, Y17A and E64A mutants were obtained the same way after PCR mutagenesis using primers. For the SsnA protein of *N. wadsworthii* DSM22245, the gene *H3L96_03690* was amplified by PCR and cloned as previously described. For *N. elongata* subsp. *glycolytica* ATCC 29315 and *Wolbachia pipientis* wMel_ZH26, the genes corresponding to *ssnA*, *NELON_RS07125*, and *FRT61_01760*, were directly synthesized with codon optimization for *E. coli* in pET15-b(+) before purification. Different purification methods have been used depending on the properties of the proteins of interest.

Production of recombinant SsnA from *N.meningitidis* 8013 2C4.3 and *N. elongata subsp. glycolytica* ATCC 29315 was performed in *E. coli* BL21(DE3) in LB medium. At OD600 of 0.6-0.8, 1 mM IPTG was added, and the culture was continued for 4 h at 37 °C. The bacteria were collected, and the pellets were resuspended in phosphate-buffered saline (PBS) at pH 8, supplemented with 10mM imidazole and 100µM PMSF. The bacteria were lysed by sonication, and the lysates were clarified by centrifugation. For SsnA from *N. wadsworthii DSM22245* and *Wolbachia pipientis,* inductions were done in *E. coli* BL21(DE3) in M9 medium with 1mM $MgCl_2$, 2% casaminoacids and 0.2% glucose. At OD600 of 0.6-0.8, 100 µM IPTG was added, and the culture was continued for 16-18h at 14 °C. The bacteria were collected, and the pellets were resuspended in 50mM Tris-HCl (pH 7.5), 100mM NaCl, 10mM imidazole and 100µM PMSF. The bacteria were lysed with a French press, and the lysates were clarified by centrifugation. The proteins were purified by chromatography on Ni-NTA resin (Fisher). Elution of good purity his-tagged proteins was performed with 300 or 500 mM imidazole and were then stored at -80°C in 50% glycerol.

Microscale thermophoresis (MST)

Purified proteins were dialysed overnight at room temperature in 20mM Tris pH8, 3mM EDTA, 300mM Arginine, 250mM ammonium sulfate, then concentrated using Amicon centrifugal devices (Sigma) with a MWCO of 3 kDa. Proteins were then diluted to working concentrations in a MST buffer containing 10mM Tris, 0.05% Tween-20, 100mM Arg, 250mM ammonium sulfate, 3mM EDTA, 100mM NaCl. Cy5-labelled oligos were used as substrates (Supplementary Data 8).

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