Functional genomics of *Neisseria meningitidis* pathogenesis

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The pathogenic bacterium *Neisseria meningitidis* is an important cause of septicemia and meningitis, especially in childhood[1]. The establishment and maintenance of bacteremic infection is a prerequisite for all the pathological sequelae of meningococcal infection. To further understand the genetic basis of this essential step in pathogenesis, we analyzed a library of 2,850 insertional mutants of *N. meningitidis* for their capacity to cause systemic infection in an infant rat model. The library was constructed by *in vitro* modification of *Neisseria* genomic DNA with the purified components of Tn*10* transposition[2]. We identified 73 genes in the *N. meningitidis* genome that are essential for bacteremic disease. Eight insertions were in genes encoding known pathogenicity factors. Involvement of the remaining 65 genes in meningocoocal pathogenesis has not been demonstrated previously, and the identification of these genes provides insights into the pathogenic mechanisms that underlie meningococcal infection. Our results provide a genome-wide analysis of the attributes of *N. meningitidis* required for disseminated infection, and may lead to new interventions to prevent and treat meningococcal infection.

The publication of the complete genome sequence of two *Neisseria meningitidis* isolates has provided a comprehensive profile of the genetic complement of this important pathogen[3,4]. However, the genome sequences emphasize inadequacies in our understanding of meningococcal pathogenesis. For example, it is not known which of the 2,158 *N. meningitidis* genes are involved in the disease process, and a substantial proportion of the open reading frames (ORFs) have no known function. Therefore, methods for high-throughput analysis of gene function are needed to exploit the information from the genome projects.

We used signature-tagged mutagenesis[5] (STM) of *N. meningitidis* to identify genes essential for septicemic infection. In STM, individual mutants are tagged with unique sequence identifiers, allowing large numbers of mutants to be analyzed simultaneously. However, for STM it is necessary to construct libraries of insertional mutants, so far a limitation in studying *N. meningitidis*. We successfully accomplished mutagenesis using a new method in which *Neisseria* DNA is modified *in vitro* using purified components of Tn*10* transposition. As *N. meningitidis* efficiently takes up exogenous DNA (ref. 6), the modified alleles are then introduced into *N. meningitidis* by transformation. We screened the mutants for their ability to cause systemic infection. Here we identified 73 genes involved in septicemic disease, including 16 of previously unknown function.

We used pSTM115 (Fig. 1*a*) as the transposon donor for *in vitro* mutagenesis. We included 96 pSTM115 derivatives, each containing unique signature tags (fig, 1*b*), in 96 separate transposition reactions. The modified genomic DNA was repaired, and returned to the host by transformation. We selected 30 mutants from each reaction, resulting in a library of 2,880 mutants (30 pools × 96). To determine whether Tn*10* insertion occurs at diverse sites, we assessed 40 transformants from a single transposition reaction by Southern blot analysis. Each had a single, distinct Tn*10* insertion (Fig. 1*c*). To establish whether Tn*10* integration was stable during systemic infection of infant rats, we compared the hybridization patterns of six mutants before and after passage through rats. We obtained identical hybridization patterns before and after infection (Fig. 1*d*).

Pilot experiments established that infant rats given 5×10^6 colony-forming units (CFU) intraperitoneally had sustained bacteremia for 32–48 hours, with peak levels reaching 1×10^6–1×10^7 CFU/ml at 18 hours (Fig. 1*e*). We used a two-stage strategy to identify attenuated mutants (fig. 1*f*). We screened 2,850 mutants in duplicate, in pools of 95 mutants, at a total dose of 1×10^7 CFU. We recovered bacteria from the blood of infected rats 20–24 hours later. Using comparative hybridization of the tags in the inoculum with those in bacteria recovered from the rats, we identified 234 putatively attenuated mutants; results from rats given the same inoculum were highly reproducible.

Certain cell surface structures expressed by *N. meningitidis* are involved in pathogenesis and are subject to high-frequency, 'on–off' switching[7]. Thus, the failure of a mutant to establish systemic infection might result from a phase variation event. We used a second round of verification to exclude this possibility. We backcrossed each mutation into the parental strain, and tested the newly constructed mutants in STM pools; 108 mutants had reduced hybridization signals in both rounds of screening.

We determined the nucleotide sequence of regions flanking the transposon in the 108 attenuated mutants. Each transposon was inserted at a distinct site, although in some cases more than one mutant had a transposon in the same gene. Thus, 73 ORFs contained transposons, and 11 insertions were located in intergenic regions. To confirm their attenuated phenotype, we tested 22 mutants in direct competition with the wild-type isolate for their ability to cause systemic infection. All mutants had substantially attenuated survival in the host.

The identification of mutants with insertions in genes encoding previously defined virulence determinants validates the approach used in the study. These mutants were affected in the biosynthesis of capsular polysaccharide, lipopolysaccharide and iron acquisition molecules. Involvement of the remaining 65 genes identified have not been previously shown to be involved

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TABLE 1   Characterization of attenuated mutants identified by STM

Gene	Mutant	Predicted function	NMB	NMA	NG	C.I.
Known virulence determinants						
lipB	5A8	Capsule polysaccharide modification	0083	0185	_	
siaD	5E11	Polysialyl transferase	0067		_	
lgtA	3E7	Lacto-N-neotetraose biosynthesis transferase	1929	0524	+	<0.001
rfaF	23A10	ADP-heptose LPS heptosyltransferase II	1527	1727	+	0.105
rfaK	7C3	α-1-2-N acetylglucosamine transferase	1705	1959	+	0.012
exbB	18C4	Biopolymer transport protein	1729	1984	+	0.032
exbD	2C6	Biopolymer transport protein	1728*	1983	+	
tonB	9B2	Iron transport	1730	1985	+	
Transport and binding proteins						
ptsH	2C9	Phosphocarrier protein	2045	0391	+	
ptsI	7A6	Phosphoenolpyruvate protein phosphotransferase	2044	0392	+	
	3D9	Oxalate/formate antiporter, putative	1362	1574	+	0.032
	7A1	ABC transporter	1240	1409	+	
	24G4	ABC transporter	1612	1811	+	0.011
	5E5	Na$^+$/H$^+$ antiporter	0536	0715	+	
	6E5	Transporter, NadC family	0792	1723	+	
	5C3	Chloride channel related protein	2006	0434	+	0.289
	12B3	GTP-binding protein, putative	1838	0618	+	<0.001
	9B10	L-lactate permease, putative	0543	0722	+	
	15H10	Putative transporter	1515	1715	+	
Amino acid biosynthesis						
aroB	1E9	3-dehydroxyquinate synthase	1814	0647	+	
aroC	5E8	Chorismate synthase	1680	1939	+	
aroD	7C10	3-dehydroxyquinate dehydratase	1446*	1659	+	
aroE	4C11	Shikimate 5-dehydrogenase	0358	2129	+	<0.001
aroG	1F12	Phospho-2-dehydro-3-deoxyheptonate aldolase	0307	2180	+	
fhs	14C8	Formate tetrahydrofolate ligase	1839	0617	+	
gdhA	22B3	NADP-specific glutamate dehydrogenase	1710	1964	+	
ilvD	3H1	di-hydroxy-acid dehydratase	1188	1361	+	
ilvI	10E2	Acetolactate synthase III, large subunit	1577*	1766	+	0.312
metF	16G5	5,10-methylenetetrahydrofolate reductase	0943*	1139	+	
metH	6G8	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	0944*	1140	+	<0.001
Cell envelope						
ampD	11C1	N-acetyl-anhydromuramyl-l-alanine amidase	0668	0867	+	<0.001
mpl-1	18A6	UDP-N-acetylmuramate:L-alanyl-gamma-D-glutamyl-meso-diaminopimelate ligase	1145	1356	+	

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in *N. meningitidis* pathogenesis (Table 1), and therefore provide new insights into the basis of meningococcal septicemia (Table 1).

Bacteria have evolved sophisticated mechanisms to sense and respond to specific environmental cues. However, nothing is known about the regulatory networks governing gene expression during meningococcal pathogenesis. Two genes were identified (*ntrY* and *hfq*) that potentially affect transcription at many loci. NtrY is a putative sensor of a two-component regulator, NtrX/NtrY (ref. 8), whereas Hfq is related to host factor 1, which influences mRNA stability in *Escherichia coli*[9]. The recognition that *hfq* and *ntrY* are required for meningococcal sepsis provides a starting point for investigating the coordinated regulation of gene expression during pathogenesis.

Seven mutants have insertions in genes responsible for integrity of the cell envelope, including four involved in peptidoglycan metabolism, the target of β-lactams, the main agents used to treat meningococcal infection. Here we have identified many genes involved in amino-acid biosynthesis, including five in the shikimate pathway, which has been widely exploited for vaccine development[10,11]. We also identified ORFs encoding sur-face-associated proteins with potential transport functions. Two putative ATP-binding cassette transporters were required for invasive infection. One of these (NMB1612) was closely related to the histidine-binding component of a transporter in *E. coli*[12]. The substrate for the other ATP-binding cassette transporter (NMB1240) is unknown.

Although the positions of attenuating mutations were widely distributed throughout the serogroup B *N. meningitidis* genome, we identified two clusters. The first contained insertions in three genes responsible for iron transport (NMB1728–1730), and one upstream of NMB1988 encoded an iron-regulated outer membrane protein. A second group of eight mutants (NMB1954–2006) included insertions in four genes of unknown function.

Comparison of the two completed N. meningitidis genome sequences showed that 8.8% of the serogroup B ORFs have no homolog in serogroup A (ref. 4). Of the 73 ORFs we identified, four were not present in serogroup A. Three encode proteins of unknown function (Table 2), whereas the other (NMB067) is necessary for capsular polysaccharide biosynthesis in serogroup B only. Most ORFs required for N. meningitidis bacteremia have

		Table 1 continued				
Gene	Mutant	Predicted function	NMB	NMA	NG	C.I.
mltB	23C10	membrane-bound lytic murein transglycosylase B	1279	1488	+	
pbp3	8G3	Penicillin-binding protein 3	1797	0665	+	0.027
rfaE	4G7	Putative ADP-heptose synthase	0825*	1034	+	
	20D12	Apolipoprotein N-aclytransferase, putative	0713	0918	+	
	4H7	Cell-binding factor, putative	0345	2142	+	
Protein synthesis						
metG	3C8	Methionyl-tRNA synthetase	0030	0275	+	
rsmB	23G7	16S RNA methyltransferase	0112	0162	+	
tufA	22A12	Translation elongation factor, Tu	0139	0134	+	
	8D6	Putative RNA methylase	1348	1560	+	<0.001
Transcription						
crhA	17G2	Putative DEAD box RNA helicase	1422	1634	+	
pnp	4A7	Polyribonucleotise nucleotidyl transferase	0758	0969	+	
rnc	15E4	Ribonuclease III	0686*	0888	+	
Energy metabolism						
fda	15G12	Fructose-1,6-biphosphate aldolase	1869	0587	+	
grxC	25H8	Glutaredoxin 3	1790*	0673	+	<0.001
Regulatory functions						
hfq	15H3	Host factor-I protein	0748	0961	+	
ntrY	6G12	Nitrogen regulation protein	0114	0160	+	
Cellular processes						
dprA	13E7	DNA processing chain A	0116	0158	+	
Biosynthesis of cofactors						
pabB	25E10	p-Aminobenzoate synthetase	1970	0477	+	
Cell division						
ispA	4E3	intracellular septation protein A	0342	2145	+	
Fatty acid and phospholipid metabolism						
dgkA	23C7	Diacyl glycerol kinase	1558	1746		<0.001
Purine, pyrimidine, nucleosides, and nucleotides						
galU	22B6	UTP-glucose 1 phosphate uridylyl transferase	0638	0848	+	0.184
purL	4A8	Phosphoribosyl formylglycinamide synthase	1996	0445	+	
Unknown:general						
dedA	23H5	DedA protein, putative	1689		+	
	6B5	Pqi protein	1671*	1929	+	
	12G3	AcrA-AcrE family protein	0548*	0728	+	0.300

NMA and NMB, ORFs with the highest level of homology to sequences flanking the transposon insertion site in serogroups A and B *N. meningitidis* genomes, respectively. NG, a + denotes the presence of sequences with significant homology (BLASTN e<10⁻⁵) in the partial *N. gonorrhoeae* genome. C.I., competitive index. *, transposon insertion may have polar effects.

related sequences in the partial N. gonorrhoeae genome. This is not unexpected, given the genetic relatedness of these species, and that N. gonorrhoeae can occasionally cause disseminated infection. To determine whether the genes of unknown function identified by STM are conserved, we examined their distribution in complete genomes of other organisms. Six ORFs did not have homologs (BLASTP e<10⁻⁵) in the other microbes examined, whereas four were conserved across bacterial genera (Table 2).

Polar effects on downstream genes can complicate the analysis of transposon mutants. To establish whether the transposon insertions are likely to have polar effects, we examined the serogroup B genome to predict whether the transposon is located in a potential operon. Polar effects were possible in 14 mutants, but as most operons contain groups of functionally related genes, these insertions remained informative.

We used a genome-wide functional analysis of *N. meningitidis* to identify genes required for septicemia. The approach differs fundamentally from the sequence-based methods that have been used to compile lists of 'pathogenicity genes' in this bacterium[4]. Such lists inevitably contain discrepancies that arise from subjective views of definitions and of the strength of evidence supporting the involvement of a gene product in pathogenesis. Functional approaches to gene identification have the advantage of being unbiased and less reliant on subjective interpretations.

It was important to confirm the attenuating mutations with a second screen. A notable feature of *N. meningitidis* is its abundance of repeated sequences that can mediate phenotypic diversity[13]. For example, expression of capsular polysaccharide, a known virulence determinant, is phase-variable because of a tract of polycytidine residues in *siaD* (ref. 14). Therefore, a mutant initially identified might be attenuated as a result of phase variation and not of transposon insertion. This was true in more than half the mutants originally identified, emphasizing the need to confirm the linkage between transposon insertion and the attenuated phenotype.

We identified several genes encoding current vaccine candidates, including lipopolysaccharide, capsular polysaccharide and iron acquisition pathways[15–17]. A 'bio-informatic survey' of the *N. meningitidis* genome was recently used to identify proteins potentially located on the surface of the bacterium[18]. About 25% of all gene products were predicted to be expressed
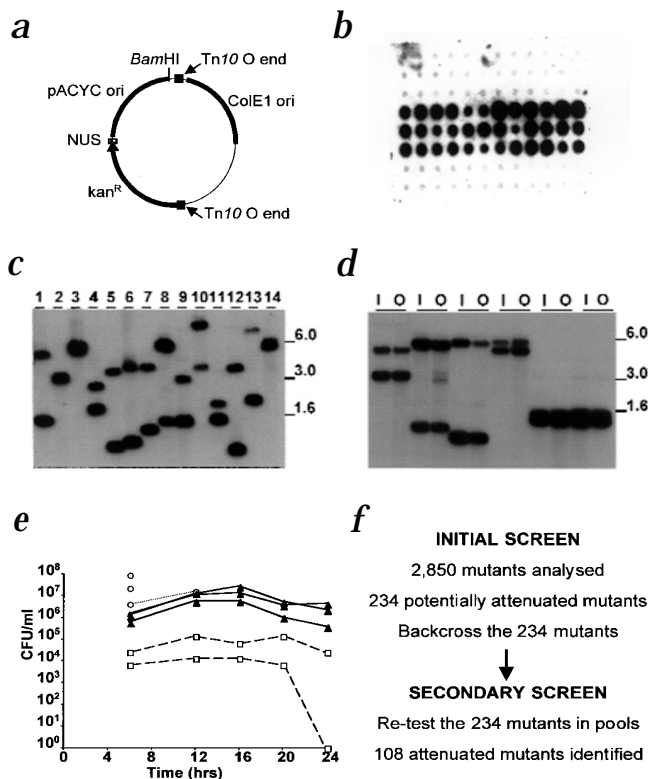
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Fig. 1 Mutagenesis of *N. meningitidis* and *in vivo* screening. *a* , The insert of pSTM115 includes the 70-base-pair 'outside (O) ends' of Tn*10* flanking the kanamycin resistance marker (kan^R), a copy of *Neisseria* DNA uptake sequences (NUS) and the origin of replication (ori) from pACYC184. *b*, PCR blots were probed with tags amplified from the middle three rows of 96 pSTM115-derivatives in a microtiter plate. *c,* Southern blot analysis of *N. meningitidis* mutants. A single integration of the transposon reaction generates two hybridizing bands because of a *Cla*I site in the kanamycin resistance cassette; doublets are present in lanes 2, 3 and 14. *d*, The hybridization pattern obtained from six mutants before (I) and 20 h after (O) inoculation of 5-day-old infant rats. Right margin (*c* and *d*), molecular sizes (1-kb ladder). *e*, The level of bacteremia after intraperitoneal inoculation of rats with bacteria. Inocula: 5×10^7 CFU (○), 5×10^6 CFU (▲) or 5×10^5 CFU (□).

on the surface, and more than 300 have been evaluated as potential vaccines (even though there is no indication whether the genes are expressed during pathogenesis). Our functional approach identified a much smaller subset of 73 genes, of which a substantial proportion encode products predicted to be located on the surface. This may form the basis of a more-stringent selection of targets for vaccine development.

A 'theme' emerging from the genome sequences is the considerable proportion of genes of unknown function[19]. Although sequencing shows the presence of these genes, it gives no indication about their involvement in the infectious process. Our work has identified attenuating mutations in 16 genes of unknown function. The functional analysis of these genes can now be addressed through phenotypic analysis of the available mutants. The profile of attenuated mutants includes many with defects in genes encoding both current drug and vaccine targets. A more-complete understanding of the involvement of the 16 genes of unknown function identified here may lead to the development of interventions against meningococcal infection.

Methods

Bacterial strains and growth. C311⁺ is an ET-5, serogroup B *N. meningitidis* isolate from a patient with invasive meningococcal infection[20]. *N. meningitidis* was grown on brain–heart infusion medium with 5% Levinthal's supplement. *E. coli* strains were propagated on Luria Bertani media. Kanamycin was added to solid media as required at concentrations of 75 and 50 µg/ml for *N. meningitidis* and *E. coli*, respectively.

In vitro transposition and insertion site characterization. Details of the *in vitro* transposition reaction are forthcoming (C.M.T., manuscript in preparation). The pACYC184 origin of replication in pSTM115 was used to isolate the insertion site by marker rescue. Nucleotide sequencing used the dye-termination method (Perkin Elmer, Norwalk, Connecticut) with primers NG62 (5'–TTGGTTAATTGGTTGTAACACTGG–3') or NG99 (5'–ATTCTCAT-GTTTGACAGCG–3'). Homology searches were performed against protein databases (http://www.ncbi.nlm.nih.gov/), including the serogroup A and B *N. meningitidis* and the *N. gonorrhoeae* genome sequences (http://www.sanger.ac.uk/ Projects/N_meningitides and http://www.tigr.org, and http://dna1.chem.ou.edu/gono.html, respectively).

Tag amplification, cloning and Southern blot analyses. Hybridizations and preparation of dot blots were performed as described[5], except that tags were amplified with primers NG13 (5'–ATCCTACAACCTCAA-GCT–3') and NG14 (5'–ATCCCATTCTAACCAAGC–3'), and PCR products, rather than plasmid DNA, were fixed onto membranes. Oligonucleotides S1 (5'–AA-GAGATTACGCGCAGACC–3') and S2 (5'–AATACG-CAACCGCCTCTC–3') anneal to sequences in pSTM115 flanking the 'signature tags' and were used to amplify a 367-base-pair product from each pSTM115 derivative. For Southern blot analysis, the kanamycin-resistance cassette from pSTM115 was labeled using the random primers method (NEB), and was used as a probe against genomic DNA digested with *Cla*I.

Animal model. For screening the STM pools, mutants were grown individually for 18 h in microtiter plates. The bacteria were pooled, then re-suspended in PBS. Wistar rats 5 d of age were inoculated intraperitoneally with 100 µl of the suspension, and were monitored for

TABLE 2	Genes encoding hypothetical proteins required for septicaemic disease									
Mutant	NMB	NMA	NG	CI	HI	EC	HP	CP	BS	MJ
1D8	1954	0497	+							
1H3	0840*	1409	+	0.118	−26	−26				
2A11	1638	1892	+	<0.001	−12	−128	−50			
4B5	0317	2170	+	0.113	−13	−12				
4F1	0673*	0873	+							
5E1	1681	1940	+							
5D12	0065									
5G12	1564	1753	+			−37				
6E5	1523		+							
10A8	0188	0079	+			−24				
11G4	1971	0476	+		−24					
12B1	1694	1952	+	<0.001		0	−61	−43	−41	−19
14G3	0734	0944	+							
18D10	1596*									
28A4	0572*	0755	+							
28C12	0183	0084	+		−59	−63	−19	−20	−22	

NMA and NMB, ORFs with the highest level of homology to sequences flanking the transposon insertion site in serogroups A and B *N. meningitidis* genomes, respectively. NG, a + denotes the presence of sequences with significant homology (BLASTN e<10⁻⁵) in the partial *N. gonorrhoeae* genome. C.I., competitive index. *, transposon insertion may have polar effects. HI, *Haemophilus influenzae*; EC, *Escherichia coli*; HP, *Helicobacter pylori*; CP, *Chlamydia pneumoniae*; BS, *Bacillus subtilis*; MJ, *Methanococcus jannaschii*. The e-value for each homolog is shown.

48 h. To establish the competitive index of a mutant, wild-type and mutant bacteria were grown for 18 h on solid media and collected into PBS, and rats were inoculated with a 1:1 ratio of mutant to wild-type cells in a total inoculum of 5×10^6 CFU. The proportion of mutant (kanamycin-resistant) to wild-type (kanamycin-sensitive) bacteria was determined by plating replicate samples to media with or without added antibiotic.

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