Analysis of the Regulated Transcriptome of *Neisseria meningitidis* in Human Blood Using a Tiling Array

Elena Del Tordello, Silvia Bottini, Alessandro Muzzi, and Davide Serruto

Novartis Vaccines and Diagnostics, Siena, Italy

Neisseria meningitidis is the major cause of septicemia and meningococcal meningitis. During the course of infection, the bacterium must adapt to different host environments as a crucial factor for survival and dissemination; in particular, one of the crucial factors in *N. meningitidis* pathogenesis is the ability to grow and survive in human blood. We recently showed that *N. meningitidis* alters the expression of 30% of the open reading frames (ORFs) of the genome during incubation in human whole blood and suggested the presence of fine regulation at the gene expression level in order to control this step of pathogenesis. In this work, we used a customized tiling oligonucleotide microarray to define the changes in the whole transcriptional profile of *N. meningitidis* in a time course experiment of *ex vivo* bacteremia by incubating bacteria in human whole blood and then recovering RNA at different time points. The application of a newly developed bioinformatic tool to the tiling array data set allowed the identification of new transcripts—small intergenic RNAs, *cis*-encoded antisense RNAs, mRNAs with extended 5' and 3' untranslated regions (UTRs), and operons—differentially expressed in human blood. Here, we report a panel of expressed small RNAs, some of which can potentially regulate genes involved in bacterial metabolism, and we show, for the first time in *N. meningitidis*, extensive antisense transcription activity. This analysis suggests the presence of a circuit of regulatory RNA elements used by *N. meningitidis* to adapt to proliferate in human blood that is worthy of further investigation.

Neisseria meningitidis is one of the major causes of sepsis and meningitis worldwide. Colonization of the upper respiratory mucosal surfaces by *N. meningitidis* is the first step in establishment of a human carrier state and then of invasive meningococcal disease. After the colonization step, *N. meningitidis* can cross the mucosal surfaces, enter the bloodstream, and produce a systemic infection, like sepsis. Bacteria may also translocate from the bloodstream across the blood-meningeal barrier, proliferate in the cerebrospinal fluid, and cause meningitis. The ability to cause invasive disease depends on environmental factors, meningococcal virulence factors, and lack of a protective immune response (67). During the transition from colonization to an invasive bloodstream infection, *N. meningitidis* must adapt to changing environments and host factors; the study of how the bacterium interacts with host factors is fundamental to understanding meningococcal pathogenesis and to setting up strategies to prevent the disease. The mechanisms of meningococcal colonization of the human nasopharyngeal epithelium have been widely reported (7), while those of *N. meningitidis* translocation across the blood-brain barrier have only recently been investigated (12, 51). Furthermore, a crucial factor in meningococcal pathogenesis is the ability of *N. meningitidis* to survive and grow in human whole blood, causing septicemia. In fact, even in the development of meningitis with low-grade meningococcemia and no shock symptoms, the bacterium must survive and multiply in blood to reach the blood-brain barrier and enter the subarachnoid space. To survive, *N. meningitidis* must adapt to the blood environment, and to do that, the bacterium has evolved molecular and cellular mechanisms to protect itself from the host immune system and to exploit the host to obtain nutrients. Human whole blood has been used as an *ex vivo* model of sepsis for studying the pathogenesis of *N. meningitidis* in terms of complement activation, cytokine production, and immunity (19, 27, 41, 55, 69), as well as the pathogenesis of other bacteria, including *Listeria monocytogenes* and group A and group B *Streptococcus* species (15, 37, 64). Using an *ex vivo* model of

bacteremia and a time course oligonucleotide microarray experiment, we have recently demonstrated that, during blood infection, *N. meningitidis* alters the expression of ~30% of the genome, and major dynamic changes were observed in the expression of transcriptional regulators, transport and binding proteins, energy metabolism, and surface-exposed virulence factors (12).

In *N. meningitidis*, as in many other pathogenic bacteria, the virulence factors need to be tightly and rapidly regulated at the gene expression level in order to control the various steps of pathogenesis. Traditionally, this regulation has been credited to the activity of transcriptional factors, but studies over the past few years have largely demonstrated the expression in bacteria of RNA regulatory molecules (2, 48, 53, 64). The regulatory RNAs constitute a heterogeneous group of molecules often acting as quick mechanisms of regulation. The majority of them modulate the expression of target genes by base pairing with the target mRNA and can be transcribed either as *cis*-encoded antisense RNAs (as-RNAs) from the opposite strand or as *trans*-encoded small RNAs (sRNAs) from physically unlinked loci. *trans*-acting small RNAs generally act by short and imperfect target pairing, often requiring RNA chaperons, such as the Hfq protein, while *cis*-antisense RNAs usually show extensive sequence complementarity with the target mRNA transcribed from the opposite strand (46). In addition, bacterial mRNAs can possess an untranslated region (UTR) at the

Received 13 June 2012 Accepted 6 September 2012

Published ahead of print 14 September 2012

Address correspondence to Alessandro Muzzi, alessandro.muzzi@novartis.com, or Davide Serruto, davide.serruto@novartis.com.

Supplemental material for this article may be found at <http://jb.asm.org/>.

Copyright © 2012, American Society for Microbiology. All Rights Reserved.

doi:10.1128/JB.01055-12

5' and/or 3' end with a posttranscriptional regulatory function in the control of the expression level of the mRNA itself (16).

In this work, we used a customized tiling oligonucleotide microarray to define the changes in the whole transcriptional profile of *N. meningitidis* in a time course experiment of *ex vivo* bacteremia, with the aim of identifying regulatory RNAs differentially expressed during this step of meningococcal pathogenesis. Using this approach, a set of small intergenic RNAs, antisense RNAs, and mRNAs with extended 5' and 3' UTRs were identified as differentially expressed during incubation in human blood. In addition, we identified sets of adjacent coregulated genes that could be part of the same operon. This analysis suggests the presence of a circuit of regulatory RNA elements used by *N. meningitidis* to adapt to proliferate in human blood and opens the way for further characterization of these transcripts as new factors and key elements in the pathogenesis of meningococci.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Serogroup B *N. meningitidis* strain MC58 was grown on gonococcus (GC) medium agar (Difco) or in GC liquid broth at 37°C in 5% CO₂. For the experiment on time course incubation in human blood, samples were treated as previously reported (12). Briefly, human whole blood was collected from three healthy volunteers by using heparin (at a concentration of 5 U/ml) and used for the sole purpose of growing bacteria. Heparin is commonly used in blood models of *Neisseria* infection (26, 27), and while heparin has been reported to affect complement activation (39), the concentration of 5 U/ml used in this work has been reported not to have an inhibitory effect. Moreover, heparin is not known to alter gene expression in *Neisseria*. Bacterial loads in patients with fulminant disease can reach up to 10⁹ bacteria/ml (9, 17, 44). In order to prepare the inoculum for use in blood, *N. meningitidis* was grown overnight on GC agar plates and cultured in GC broth to early exponential phase (optical density [OD], 0.5 to 0.6). Approximately 10⁸ bacteria were pelleted by centrifugation at 4,000 rpm for 5 min and resuspended in an equal volume (1 ml) of freshly isolated human blood maintained at 37°C. Human whole blood infected with bacteria was incubated for 0, 30, 60, and 90 min at 37°C and 5% CO₂, with gentle shaking to avoid sedimentation. Samples were then treated with RNA Protect bacterial reagent (Qiagen) immediately after adding bacteria to human whole blood (time zero [*t*₀]) and for each time point to 90 min. Cells were harvested by centrifugation and stored at -80°C until bacterial RNA isolation. Each time point was represented by three incubation samples from which RNA was purified separately. CFU counts were obtained for *N. meningitidis* cultures immediately before time course initiation (time zero) and after 30, 60, and 90 min of incubation, and the curves showed the same growth rate of bacteria in blood for all the donors (data not shown). The time course experiments were performed independently with three different blood donors.

For the TOPO-TA cloning strategy, *E. coli* strains were cultured in Luria-Bertani (LB) agar or LB broth at 37°C, and ampicillin was added at a final concentration of 100 µg/ml. White/blue selection was performed by adding IPTG (isopropyl-β-D-thiogalactopyranoside) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) to LB agar according to the manufacturer's instructions.

Isolation and enrichment of bacterial RNA. RNA isolation was performed as previously described (12). Briefly, the samples were incubated with 5 volumes of erythrocyte lysis (EL) buffer (Qiagen) for 15 min on ice and centrifuged at 4°C and 4,500 rpm for 6 min. Total RNA (bacterial and eukaryotic) was isolated by enzymatic lysis using lysozyme (Sigma) at 0.4-mg/ml final concentration, vortexed, and incubated at room temperature for 5 min. RNA isolation was completed with the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. DNA contamination was avoided by on-column treatment with an RNase-Free DNase Set (Qiagen) and posttreatment with recombinant DNase I (Roche) accord-

ing to the manufacturers' instructions. Absence of bacterial DNA was confirmed by PCR with primers specific for *NMB1591* (see Table S7 in the supplemental material). The RNA concentration and integrity were assessed by measurement of the A₂₆₀/A₂₈₀ ratios and electrophoretic analysis with an Agilent 2100 Bioanalyzer (Agilent Technologies). Finally, three total-RNA aliquots corresponding to each time point were pooled and used for the bacterial RNA enrichment procedure. Total RNA obtained at this step, extracted at time zero and after 60 min of incubation, was also used for 5'-3' rapid amplification of cDNA ends (RACE) and reverse transcriptase (RT) PCR. Enrichment of bacterial RNA was performed using the MicroBEnrich kit (Ambion) according to the manufacturer's instructions. Enriched bacterial RNA was assessed by electrophoretic analysis with an Agilent 2100 Bioanalyzer. Enriched bacterial total RNA was used as the template for cRNA synthesis (amplification and labeling reaction).

RNA amplification and labeling. Enriched bacterial RNA was amplified and labeled using the MessageAmp II Bacteria kit (Ambion). The kit employs an *in vitro* transcription (IVT)-mediated linear amplification system to produce amplified RNA (cRNA). Briefly, 100 ng of total RNA from each time point was used as the template for the synthesis reaction. An initial polyadenylation step was performed. The tailed RNA was reverse transcribed (cDNA synthesis) in a reaction primed with oligo(dT) primers bearing a T7 promoter. The resulting cDNA was then transcribed with T7 RNA polymerase to generate cRNA in an *in vitro* reaction for 14 h at 37°C. The cRNA was labeled by including Cy3-CTP (Cy3) and Cy5-CTP (Cy5) (Perkin Elmer, Boston, MA) nucleotides in the IVT reaction. This protocol allowed us to obtain labeled strand-specific cRNA. The cRNA was then fragmented in fragmentation buffer (Agilent Technologies) at 60°C for 30 min before hybridization. Competitive hybridizations were conducted with 825 ng of Cy3-labeled reference cRNA (bacteria in contact with human whole blood at time zero) versus 825 ng Cy5-labeled cRNA from each time point (30, 60, and 90 min). cRNAs were hybridized onto the microarray slides for 17 h at 65°C, washed, and scanned with an Agilent scanner following the Agilent Microarray protocol.

Microarray design and analysis. The design of the tiling array was mainly performed with ArrayDesigner v.4.2 using a specific procedure for 60-mer probe selection, and the chip was produced with the Agilent *in situ* technology. In detail, the chromosome of *N. meningitidis* strain MC58 (GenBank accession no. AE002098) was subdivided on the basis of its open reading frame (ORF) predictions into coding and noncoding regions, considering small ORFs (<300 bp) to be noncoding, on both the forward and reverse strands. Noncoding regions were the basis for the selection of tiling probes of 60 bp, shifted 10 bp. No cross-homology filtering was imposed because the coverage of the chromosome was a priority and cross-homology was checked *a posteriori* by BLAST. We used an ArrayDesigner algorithm to check the sequences and structures of the probes, rejecting the problematic ones. Instead, to represent coding portions of the chromosome (predicted ORFs with lengths of >300 bp), we used the nontiling probes already designed for a gene-based array described previously (12) (Array Express Design ID, A-MEXP-1957).

Finally, 36,869 probes for noncoding portions of the chromosome and 6,877 probes for coding portions were selected, and in addition, control probes specific to the Agilent technology were included in the final 4,000-by-44,000 custom chip design.

After data acquisition, slide normalization was performed by a lowess (locally weighted scatterplot smoothing) algorithm as implemented by the Agilent Feature Extraction software v.9.5.3. Before any other analysis, we computed an average of replicated probe signals [$M = \log_2(\text{Cy5}/\text{Cy3})$ and $A = \log_{10}(\text{Cy3} \times \text{Cy5})/2$] within each slide. Then, *M* probe signals of the experimental replicas at each time point were compared by principal-component analysis (PCA) as described below. The average signals at each time point were computed by merging the slides of the different donors.

PCA comparison of the gene-based array and the tiling array. We applied the same PCA procedure described previously (28). The expression profile of each probe during the time course in each blood donor was

modeled by using PCA, and then, each profile was expressed as a linear combination of the dominant principal components (PCs). Hence, the data were projected on each PC, and that projection was the score for that probe in each PC. The score for a probe on a PC is the correlation between the probe and the PC. Comparing the scores is equivalent to assessing the similarity of temporal expression profiles. The use of PCA allows the identification of probes with different expression profiles under different conditions by the examination of the score distribution: probes whose expression does not change under different conditions will have similar scores; conversely, probes with different expression profiles under different conditions will have different scores. A hypothesis test based on the distribution of the Mahalanobis distances was used to evaluate the significance of the differences between the scores through calculation of the *P* value. The Mahalanobis distance is a common metric used with PCA; it is used to find the distance from each point to the centroid. Probes whose Mahalanobis distances are large have an unlikely behavior and small *P* values (28), and they are defined as noisy. Four control hybridizations for the tiling array were analyzed to identify these noisy probes: 3 hybridizations, 1 for each blood donor (comparing the *N. meningitidis* RNA extracted at time zero to itself), and a DNA comparative genomic hybridization (CGH) (comparing the genomic DNA [gDNA] of *N. meningitidis* strain MC58 to itself). In each hybridization, the same condition was tested against itself. For the comparisons between different blood donors and time courses and to a previously published time course experiment analysis of *N. meningitidis* growth in human blood and based on a gene-based array (12), the experiments taken into account were as follows: 4 time course experiments analyzed by the gene-based array (each time course was based on meningococcal growth in blood from a different donor and recovering *N. meningitidis* RNA at time points 0, 30, 60, and 90 min after blood contact) and 3 time course experiments analyzed by the tiling array (using the same experimental procedure and 3 different blood donors). Since the two experiments have two different kinds of chip design, only the probes common to the two chip layouts were selected, for a total of 4,432 probes.

Differentially expressed transcript identification by the chipSAD method. The second level of the analysis was done by taking into consideration all the available probes, covering both intergenic and antisense regions. This was done with a novel probe position-based approach called the chip signal area detector (chipSAD) method. The main idea of chipSAD is to cluster contiguous, strand-specific, and intensity-correlated probes and to compute a statistical analysis at the level of these clusters in order to identify the intensity change point to obtain the segmentation of the hybridization signal in transcription units. The method can be applied to both single- and two-color microarray experiments, and in particular, it is suitable to the segmentation analysis of the log₂ ratio of the two fluorescence intensity signals Cy5/Cy3 (*M* value) and 1/2 log₁₀ of their product (*A* value). The approach is divided into two main steps: at the beginning, the probes within a non-fixed-width sliding window and having similar intensities are grouped together, making a “correlated probe region” (CPR). Then, for each point along the genomic coordinate, a modified *t* test is computed between its right and left CPRs to detect the intensity change point. Finally, joining a close stretch of probes for which the *t* test hypotheses are maintained between two consecutive intensity change points identifies the signal areas (SAs). Type I error rate control was done by false discovery rate (FDR) statistics based on the estimation of the *q* values for each *t* test (57). The expression level, *M*, of each signal area was computed by using the pseudomedian or Hodges-Lehmann estimator (23, 49). The final step was to compare the signal areas to the ORF prediction of the published genome. The goal of this comparison is to classify the putative transcriptional units, not only when they represent single ORFs, but also when they can represent entire operons, antisense RNAs, mRNAs with extended UTRs, and intergenic RNAs. This classification was performed by following several steps, starting from the searching of SAS regions overlapping annotated ORFs; if the SAS and the overlapped ORF are in the same strand, the region is classified as an ORF;

otherwise, it is classified as antisense if the overlap is at least 30% of the ORF length. If the SAS overlaps more ORFs (at least 30% of each ORF length of the same strand), it is classified as an operon. If it overlaps more than one ORF in two different strands and the lack of probes between the two ORFs covers a region of less than 30 bases, it is classified as an overlapping UTR. If the SAS region does not overlap an ORF in both strands and its length is less than 800 bases, it is classified as intergenic. Finally, if the SAS region overlaps an ORF and an intergenic region (IG) and the lack of probes between the two ORFs covers a region of less than 30 bases, it is classified as a UTR. Finally, we considered relevant SAS regions differentially expressed when they showed a *P* value of less than 0.05 and a pseudomedian *M* value greater than 0.92, or less than −0.92 for antisense signal areas and higher than 0.68 or less than −0.68 for the other kind of transcribed objects (these two thresholds were evaluated at 2 times the sigma width of the peak distribution of pseudomedian *M* values under the control conditions in the blood time course, time zero, for antisense and sense signal areas, respectively). The false-discovery rate estimation for each time point was performed for each class of transcription unit. Excluding time point zero, we observed similar values of the FDR of each class independent of the time point. In detail, the FDR at a *P* value of <0.05 was 2% for all the sense, antisense, and UTR areas of signal; the FDR was 0.2 to 0.6% for overlapping UTRs and 16% for intergenic areas of signal.

K-means clustering of sense and antisense transcripts. Clusters of coregulated sense and antisense transcripts were identified by a *K*-means algorithm. The antisense and sense trends were concatenated in order to obtain one curve for each pair of antisense-sense transcripts. The evaluation of the optimal number of partitions was done by performing a figure-of-merit (FOM) analysis as implemented by the TMEV software (50). FOM analysis showed that the optimal number of clusters was 10.

Sequence conservation, promoter prediction, and target prediction of the identified small intergenic RNAs. All the small-RNA transcripts detected in intergenic regions by mapping the tiling array results in the Artemis annotation environment were analyzed with regard to location, features, potential function, and previous identification in the literature. The set of small RNAs was checked for correspondence with known tRNAs (<http://cmr.jcvi.org/>). Then, for each intergenic small-RNA transcript, several analyses were performed. The conservation of the sequence was checked among different species and strains used as references for the neisserial population: MC58 (*N. meningitidis* group B), Z2491 (*N. meningitidis* group A), FAM18 (*N. meningitidis* group C), *N. meningitidis* alpha14, *Neisseria gonorrhoeae* FA 1090, and *Neisseria lactamica* 020-06. The sequences were analyzed and aligned using Geneious software.

For each small RNA, the presence of a putative promoter at the 5′ end (http://www.fruitfly.org/seq_tools/promoter.html) and a Rho-independent terminator of transcription at the 3′ end (a list of *N. meningitidis* MC58 strain Rho-independent terminators is available at <http://cmr.jcvi.org/>) was checked.

In addition, the putative target mRNAs for each small RNA were determined using the TargetRNA tool (<http://snowwhite.wellesley.edu/targetRNA>).

Motif analysis of the identified UTRs with the Rfam database. For 5′ and 3′ UTR analysis, the presence of known functional RNA motifs was checked by using the Rfam database (<http://rfam.sanger.ac.uk/>).

Comparison of the identified operons with the *in silico* prediction by the DOOR algorithm. Operons that were putatively detected by the observed transcription signal were compared to an *in silico* prediction of operon boundaries in the MC58 chromosome, as reported in the Database of proKaryotic OpeRons (DOOR) (<http://csbl1.bmb.uga.edu/OperonDB/>).

Simultaneous mapping of 5′ and 3′ ends of RNA molecules (5′-3′ RACE). The basis of the 5′-3′ RACE approach is the simultaneous mapping of 5′ and 3′ RNA ends by RACE using circularized RNAs (the protocol was adapted from that of Toledo-Arana et al. [64]). Ten micrograms of total RNA was split into two aliquots. Both aliquots were incubated for

1 h at 37°C with or without tobacco acid pyrophosphatase (TAP) (Epicentre Biotechnologies) in the corresponding buffer (20 U of TAP enzyme in a 50- μ l reaction volume). This step allows the discrimination of a 5' end generated by transcription initiation from a 5' end provided by RNA processing. RNA was purified by acid-phenol and chloroform extractions and ethanol precipitation. Two hundred fifty nanograms of the TAP⁺ and TAP-treated RNAs were incubated with 20 U of T4 RNA ligase I (New England BioLabs) in the presence of 1 $\times$ RNA ligase buffer, 8% dimethyl sulfoxide (DMSO), 10 U of RNase Inhibitor, 1 U of DNase I (Roche), and RNase-free water in a total volume of 25 μ l at 17°C overnight. The ligated RNA was purified by acid-phenol and chloroform extractions and ethanol precipitation and resuspended in 10 μ l of RNase-free water. The ligated RNA was used to perform an RT-PCR using specific outward primers (see Table S7 in the supplemental material) and the SuperScript One-Step RT-PCR kit (Invitrogen). The specific outward primers were designed adjacent to one another so that the size of the PCR product obtained would correspond to the real size of the small RNA analyzed. The RT-PCR products were checked on 3% Tris-acetate-EDTA (TAE)-agarose gels, and the bands present only in the TAP⁺ reactions and corresponding to the expected size were purified using a gel extraction kit (Qiagen) and cloned using a TOPO-TA cloning kit (Invitrogen). Ten clones for each transformation were analyzed by PCR, using the same pair of primers used in the RT-PCR, and the plasmids containing inserts of the expected size were sent for sequencing, using M13 forward and reverse primers.

RT-PCR to detect antisense RNAs, mRNAs with 5' and 3' UTRs, and operons. Specific RT-PCR was performed using total RNA extracted at t_0 (for downregulated transcripts) and t_{60} (for upregulated transcripts) of incubation in human blood. Total RNA was previously treated with DNase I (Roche) and checked for the absence of residual genomic DNA. Reverse transcription was performed using Superscript III (Invitrogen), and specific primers (see Table S7 in the supplemental material). For antisense RNA detection, in the reverse transcription step, reverse primers were designed to be complementary only to antisense RNA and not mRNA. For detection of UTRs, we used two different pairs of primers, one designed inside the coding sequence of the gene and one that matched the last probe of the transcriptional unit, which gave a signal in the tiling array analysis. For long transcripts, corresponding to operons, the reverse transcription was performed using a primer designed at the end of the transcriptional unit. Then, a PCR was run using a pair of primers to amplify the entire transcript and, when possible, pairs of primers designed inside two adjacent genes in divergent orientations in order to amplify the intergenic regions (in a long transcript, they were cotranscribed together with the genes of an operon). For all types of transcripts, 500 ng of total RNA was incubated with 1 μ l deoxynucleoside triphosphate (dNTP) mixture (10 mM each; Invitrogen), 2 pmol of gene-specific primer, 1 $\times$ first-strand buffer, 2 μ l 0.1 M dithiothreitol (DTT), 1 μ l RNaseOut (40 U/ μ l; Invitrogen), and 1 μ l Superscript III RT (200 U), according to the manufacturers' instructions. A sample without RT polymerase was added as a negative control. After reverse transcription, RT⁺ cDNA samples were treated with 1 μ l (2 U) of *E. coli* RNase H (New England BioLabs) and incubated at 37°C for 20 min. The RNase H was inactivated for 20 min at 65°C; 2 μ l of each sample (including the RT-negative control, not treated with RNase) was used for PCR with Platinum Taq Polymerase (Invitrogen) using specific primers (see Table S7 in the supplemental material). The RT-PCR products were checked on 1% TAE-agarose gels.

RESULTS

Analysis of the whole transcriptome of *N. meningitidis* in an *ex vivo* model of human bacteremia. In a previous work (12), *N. meningitidis* was incubated in freshly heparinized human blood over a time course experiment in an *ex vivo* model of bacteremia in order to study the meningococcal responses to both cellular and humoral blood components at the gene expression level by using a traditional gene-based microarray design. In the present work, the investigation was broadened to the whole transcriptome of *N.*

meningitidis, using a customized 60-mer tiling oligonucleotide microarray. The array contained probes designed in the coding sequence of the genes (also present in the microarray used in the previous work) and probes covering head to tail the antisense strand of each gene and all the intergenic regions in both strands. *N. meningitidis* was incubated in human whole blood in a time course experiment, and samples were collected at four different time points: immediately after mixing bacteria with blood (time zero, used as the reference point) and after 30, 60, and 90 min of incubation in blood at 37°C and 5% CO₂. Total RNA was extracted, and samples were enriched in bacterial RNA. Transcriptional changes throughout the course of incubation in human blood were defined by comparison of the expression levels at various time points to that at time zero. Whole venous human blood collected from three healthy donors was used for three independent biological replicates of the experiment, and the analysis was performed considering the data set resulting from each single experiment (e.g., each single donor) and also the three data sets merged.

Validation of the array and identification of new transcribed regions specifically expressed during incubation in human whole blood. We applied the PCA algorithm for the identification of noisy probes in the custom-designed *N. meningitidis* tiling oligonucleotide array. The resulting number of probes identified as noisy was 3,099 out of 43,746 (corresponding to 7% of the probes). Then, a set of 4,375 probes was also identified as having cross-homologies within the *N. meningitidis* chromosome, and they were considered nonspecific. In addition, PCA was used both to compare the reproducibilities of the different replicates of blood donor profiles during the time course and to compare our results to a previously published gene expression data set (12) based on an *N. meningitidis* gene-based oligonucleotide array. The PCA decomposition was limited to the probes that are common to the two designs, and it showed good superimposition of the probes and experiments of the two data sets (see Figure S1 in the supplemental material), confirming the consistency of the tiling array data set.

The hybridization signal of tiling arrays is quasicontinuous, and the boundaries of the transcribed regions can be investigated with higher accuracy than with traditional gene-based arrays (25, 62). To address this task, we developed and applied a new sliding-window method (chipSAD) for the analysis of both coding and noncoding portions of the genome, taking into account the variability of the probe density and their positions. The method detects the limits of differentially transcribed regions and estimates the level of significance of the signal with respect to the null hypothesis that the region is not regulated. Since the experimental procedure was based on a competitive 2-color hybridization experiment, the analysis was performed considering the *M* value, that is, the log₂ ratio between the two signals (Cy5 for each time point of incubation and Cy3 for the reference condition at time zero). The merged data set, based on the average signals of the probes, was analyzed using the chipSAD tool, and the boxes of correlated probe regions were manually inspected along the genome using Artemis software, in order to map the transcripts, considering both the coding and noncoding regions.

The transcriptional units were classified considering the genomic positions and the orientations of the probes that were part of every single unit. In this way, it was possible to identify different groups of regulated transcripts: small intergenic RNAs,

FIG 1 Summary of transcripts differentially expressed by *N. meningitidis* during incubation in human blood. (A) Number of transcripts of each type detected with the tiling array and differentially expressed during incubation in blood. (B) Numbers of the different types of UTRs detected in this study. (C) Graphical representation of the number of genes per polycistronic operon identified.

antisense RNAs, mRNAs with extended 5' and 3' UTRs, and operons (Fig. 1A). Statistics on the M values of the probes corresponding to each transcriptional unit were applied to reveal differentially expressed transcripts. If the pseudomedian (23, 49) of the M values of the unit was above a fixed threshold, set as less than -0.68 for downregulation and >0.68 for upregulation (the threshold was set up by comparing t_0 to t_0 [see Materials and Methods]) and if the t test significance statistics reported a P value of <0.05 , then the transcriptional unit was considered differentially expressed.

The genomic positions of the regulated transcriptional units showed strong correlation with the positions of known *N. meningitidis* annotated ORFs. In addition, the analysis revealed numerous signals located in intergenic regions and in the antisense strands of annotated ORFs. Overall, nearly 25% of the probes designed in the intergenic regions gave a signal above the background, and in particular, 91 intergenic transcripts were identified with a consistent signal during the time course experiment. The analysis of the antisense strand for each ORF revealed that 14.3% of the genes had a signal in the opposite strand, with 260 transcripts identified. In addition, for 88 genes, the probes designed immediately up- or downstream of the coding sequence gave a signal above the background and similar to those of the probes in the coding sequence. This means that for these genes, the transcription started upstream and/or ended downstream of the coding sequence leading to a 5' and/or 3' UTR. Finally, 141 transcriptional units contained both probes in the coding sequences of adjacent genes and probes in intergenic regions, suggesting the presence of operons. All detected transcripts are listed in Tables S1 to S4 in the supplemental material. It is important to note that the protocol used allowed only the identification of differentially ex-

pressed transcripts. The different classes of transcripts are analyzed and discussed below.

Small intergenic RNAs. In addition to expression of known coding regions, a consistent number of transcriptional units mapped in intergenic regions. The criteria used for the analysis were as follows: (i) there was no annotated ORF according to the published annotation of the genome (61), (ii) there was a higher signal level than for neighboring probes, (iii) there was a higher signal level than for the corresponding antisense strand, and (iv) there was no cross-hybridization of the probes in the unit with other regions in the genome. This screening resulted in the identification of 91 transcripts, which are listed in Table S1 in the supplemental material. They have a median length of 126 nucleotides (nt) and range from 60 nt (corresponding to a single probe) to 457 nt (in only one case was an intergenic transcript of 801 nt detected), and none of them correspond to known tRNAs. The sequences of the regulated intergenic transcriptional units were analyzed for possible homologies to known genes in other bacterial species. In 13 cases, similarity to annotated genes was revealed (see Table S5 in the supplemental material). Even if all these pieces of evidence were observed for hypothetical uncharacterized ORFs, they might suggest an alternative prediction for some of these *N. meningitidis* transcripts.

From the list of sRNAs, 8 highly regulated transcripts were selected for experimental confirmation (see Table S1 in the supplemental material) using a modified 5'-3' RACE protocol that allowed simultaneous determination of 5' and 3' ends of the RNA molecule (64). The experiments were performed using total RNA, extracted at time zero for downregulated small RNAs or after 60 min of incubation in human blood for upregulated small RNAs. The RACE results confirmed the presence of 7 small RNA molecules (called Bns, for blood-induced neisserial small RNA) in our samples, and in all cases, the results were correlated with tiling array signals (Fig. 2; see Figure S2 in the supplemental material). Moreover, the protocol allowed us to obtain the exact sequence of the RNA molecule that gave a signal in the tiling array. The sequences were analyzed with respect to their conservation among different species that were considered reference species of the neisserial population using Geneious software. In addition, for each small RNA, bioinformatic tools were used to predict the promoter, the presence of a Rho-independent transcription terminator (Fig. 2C), and the possible target mRNAs (see Table S6 in the supplemental material).

One of these sRNAs mapped in the IG between *NMB2073* and *NMB2074*. The sequence obtained with RACE amplification showed that this small RNA corresponded to NrrF, a recently identified small RNA induced under iron starvation (35, 38). The presence of NrrF in our sample was also confirmed by Northern blotting, and the expression of NrrF in human blood was comparable to that observed under *in vitro* conditions of iron starvation (see Figure S2D in the supplemental material). This result confirmed the ability of the tiling array to detect the presence of small RNAs in our samples and demonstrated that NrrF was highly expressed in an *ex vivo* model of bacteremia.

Among other transcripts identified, Bns1 (IG *NMB1563*-*NMB1564*) has a length of 77 nt and corresponds to one probe that was highly upregulated (M value, 3.6 at t_{60}) (Fig. 2A). Sequence alignment showed that it is conserved in all neisserial species analyzed, except *N. lactamica*. In the prediction of target mRNA, the best hit was *NMB0429* (see Table S6 in the supplemental mate-

rial). This gene, annotated as encoding a small hypothetical protein, was found to be part of the same operon as *prpB* (2-methylisocitrate lyase) and *prpC* (methylcitrate synthase) and was strongly upregulated in blood (see Fig. 7B; see Table S4 in the supplemental material).

Bns2 (IG NMB2062-NMB2063) has a length of 85 nt, and it is conserved in all the genomes analyzed (Fig. 2B). It has a predicted Rho-independent terminator and a promoter sequence. Interestingly, Bns2 sequence mapped in the same intergenic region of the small RNA mc05, identified by Mellin and coworkers (35) during a screening of putative Fur-regulated small RNAs.

In the intergenic region between NMB0910 and NMB0911, four upregulated probes in a single transcriptional unit met the specific criteria used. This region is part of a putative prophage in *N. meningitidis* serogroups B and C (11). RACE analysis, performed using two pairs of primers annealing in the first two or the last two probes, showed the presence of two distinct RNA molecules, named Bns3a and Bns3b (Fig. 2C). As expected, among the genomes considered, the sequences are conserved only in *N. meningitidis* group C FAM18 and *N. meningitidis* alpha14, where the prophage is present, although in a different chromosomal locus. Since the search for promoter and Rho-independent terminator sequences resulted in only one putative promoter before the first probe and a terminator inside the latter probe (Fig. 2C), the sequence was probably transcribed as a single transcript and then processed. Interestingly, Bns3b is predicted to base pair with NMB0607 (see Table S6 in the supplemental material), which codes for a protein export protein, SecD, that was not regulated in blood but was downregulated in an Hfq deletion mutant (36).

In addition, RACE analysis of total RNA extracted at time zero confirmed the expression of a downregulated small RNA, Bns4, that mapped in the intergenic region between NMB1016 and NMB1017 (see Figure S2A in the supplemental material). Bns4 was strongly downregulated during the incubation in blood, and the sequence is not conserved in *N. lactamica*. The bioinformatic analysis performed did not allow the prediction of a promoter or terminator sequence, but in the search for putative target mRNAs, the most interesting hit was the *tonB* 5' end (NMB1730) (see Table S6 in the supplemental material), which was strongly upregulated in human blood as an operon, together with NMB1728 and NMB1729 (see Table S4 in the supplemental material).

In the analysis of the new small RNAs identified, a putative consensus for Hfq binding was found in 4 of them (Fig. 2; see Figure S2C in the supplemental material). This is not surprising, since potential contact sites of Hfq in sRNAs have weak conservation at the nucleotide level (68); moreover, there are many examples of Hfq-independent sRNAs in other Hfq-expressing bacteria, like *vrnA* in *Vibrio cholerae* (54) and *symR* in *Escherichia coli* (30).

Among the expressed intergenic regions, one corresponded to

N. meningitidis transfer-messenger RNA (tmRNA) NmtmRNA1 (IG NMB1015-NMB1016) (see Table S1 in the supplemental material), which was slightly upregulated during incubation in human blood. tmRNA is a structural housekeeping RNA that binds to damaged mRNA and, in other bacterial pathogens, it is reported to have different roles; interestingly, in *Salmonella enterica* serovar Typhimurium, the corresponding deletion mutant is attenuated in various infection models (1, 29).

Small RNAs are reported to regulate gene expression both positively and negatively (46, 68). This could help to explain the diverse levels of expression of sRNAs and putative mRNA targets that we observed during incubation of *N. meningitidis* in human blood, as in some cases, sRNAs and their putative target RNAs were both up- or downregulated, while in other cases, their expression levels were inversely related (see Table S6 in the supplemental material for small-RNA predicted targets).

Antisense RNAs. In mapping the transcriptional units in the genome, nearly 14.3% of the genes had a signal in the antisense strand. In a detailed analysis, 260 antisense RNAs were transcribed and differentially expressed during incubation in human blood; of these, 27% were upregulated and 73% were downregulated (see Table S2 in the supplemental material). As for the intergenic transcripts, the sequences of the regulated antisense RNAs were analyzed for possible homologies with known genes in other bacterial species. In 7 cases, similarity to annotated genes was revealed (see Table S5 in the supplemental material). Except for AS1 and AS28, matching a modification methylase (NMCC_1934a) and *cybB* (NMCC_2052), respectively, all the matches were with hypothetical uncharacterized ORFs.

The antisense transcripts detected in this study could correspond to *cis*-encoded asRNAs, i.e., RNAs encoded in the same DNA locus as their complementary putative targets. If there is a regulatory function of asRNAs, it appears likely that the expression level of an asRNA is somehow coupled with the expression level of the corresponding mRNA. In our time course experiment, it was possible to identify different types of correlation in the expression levels between an mRNA and its corresponding antisense transcript (Fig. 3A): in 23.4% of genes, they were either up- or downregulated; in 44.8% of genes, the mRNA was not differentially expressed, while the antisense RNA could be either up- or downregulated; finally, in 31.8% of cases, the two transcripts were inversely regulated. Interestingly, in 29.5% of cases, the antisense RNA was downregulated and the corresponding mRNA was upregulated, while in only 2.3% did we observe the upregulation of asRNA and downregulation of mRNA (the list of all detected antisense transcripts and the relative expression data are reported in Table S2 in the supplemental material). In a further analysis, performed using a *K*-means algorithm, the expression profiles of antisense RNAs and the corresponding sense mRNAs were analyzed

FIG 2 Small intergenic RNAs detected by the 5'-3' RACE protocol. (a) For each small RNA, the transcriptional map obtained by the tiling array is reported. In the y axis, the plot shows the normalized *M* value (the log₂ ratio of the two channels, Cy5 for samples recovered at 30, 60, and 90 min of incubation in blood and Cy3 for the reference condition at time zero) for the positive strand (Fwd) and the negative strand (Rev). The scheme of the genomic position is in the x axis. Annotated ORFs are represented by blue arrows and detected transcripts by dashed blue lines. The signal background is delimited by dashed gray lines. Each dot corresponds to the average of the intensity signals for one probe from three independent biological repetitions. For each probe, four dots in different colors are reported, corresponding to the four time points of the time course. (b) For each small RNA, the RACE result is reported next to the 100-bp ladder. (c) The sequences determined by RACE analysis in different neisserial genomes were compared (*N. meningitidis* serogroups A, B, and C; *N. gonorrhoeae*; *N. lactamica*; and *N. meningitidis* alpha14) (nucleotides that were not conserved are shaded in gray). The sequence obtained by sequencing the RACE PCR product is boxed in orange. In the sequence, the predicted promoter (black lines), the predicted terminator (red lines), and the putative Hfq-binding site (dashed black line) are also shown. (A) Bns1 (IG NMB1563-NMB1564). (B) Bns2 (IG NMB2062-NMB2063). (C) Bns3 (IG NMB0910-NMB0911).

FIG 4 Clusters of correlation between sense mRNA and related *cis*-encoded antisense RNA expression profile dynamics. Ten clusters of the coregulated antisense and sense transcripts were obtained using a *K*-means algorithm. Each cluster regroups different combinations of the expression profiles of sense (black lines) and antisense (gray lines) RNAs. Clusters A and B collect antisense and sense profiles, both downregulated but with different shapes and amplitudes of regulation. In cluster C, both antisense and sense profiles are upregulated. Clusters D, E, and F represent downregulated antisense profiles together with unregulated sense profiles, again with different trends and amplitudes of regulation. Cluster G represents upregulated antisense profiles with unregulated sense profiles. Clusters H to J collect downregulated antisense profiles with upregulated sense profiles. Only two clusters, C and G, contain upregulated antisense transcripts, because most of the antisense RNAs were downregulated in blood.

in detail, and 10 different clusters of combinations of antisense and sense RNA expression behaviors over the time course experiment were identified (Fig. 4). The relative expression of each mRNA and its *cis*-encoded antisense RNA could be followed over a certain period of time. For example, in cluster D, asRNAs showed a rapid decrease in expression after 30 min with a subsequent stable downregulation, while in cluster F, asRNAs were slightly downregulated during the time course experiment. Similar dynamics were observed for the expression profiles of the dif-

ferentially expressed genes of *N. meningitidis* in blood analyzed in the previous study (12).

The presence of antisense RNA molecules in our samples was confirmed by specific RT-PCR (8). The reverse transcription was performed using a reverse primer specific only for the antisense RNA, followed by digestion with RNase H and a subsequent PCR with a specific pair of primers (Fig. 5A). Using this approach, 16 asRNAs were tested and confirmed in total-RNA samples ex-

FIG 3 *cis*-encoded antisense RNAs. (A) Percentages of the different types of antisense-sense RNA expression correlation with respect to the entire set of antisense RNAs detected in this study: (1) both sense and antisense RNAs are downregulated; (2) both sense and antisense RNAs are upregulated; (3) the mRNA is not differentially expressed, while the *cis*-antisense RNA is downregulated; (4) the mRNA is not differentially expressed, while the *cis*-antisense RNA is upregulated; (5) the mRNA is upregulated, while the *cis*-antisense RNA is downregulated; (6) the mRNA is downregulated, while the *cis*-antisense RNA is upregulated. (B) A subpanel of *N. meningitidis* antisense RNAs identified and differentially expressed during incubation in human blood. For each antisense RNA, the following are shown: the strand (forward for 5'-3' genomic orientation and reverse for the opposite strand); the gene to which the RNA is antisense with annotation and the function of the ORF; the start and end of the signal; the length of the signal; and the transcriptional profile of the antisense RNA and mRNA, reported as the *M* value (the log₂ ratio between the two channels, Cy5 for 30, 60, and 90 min of incubation in blood and Cy3 for the reference condition at *t*₀), if the transcript was tested by RT-PCR.

FIG 5 Detection of identified *cis*-encoded antisense RNAs by RT-PCR. (A) Scheme of the RT-PCR strategy used to confirm the presence of *cis*-antisense transcripts in total RNA extracted after incubation of *N. meningitidis* in human whole blood. The reverse transcription was performed using a primer specific only for the antisense RNA, and then a PCR was used to amplify and detect the transcript. The dashed blue arrow indicates the asRNA, the dashed black arrow the mRNA of the corresponding gene, and the red arrows the primers used for RT-PCR. (B) Examples of *cis*-encoded antisense RNAs confirmed by RT-PCR that correspond to the different types shown in Fig. 3. For each antisense RNA, the transcriptional map (a) and the RT-PCR results (b) are shown. In the transcriptional map, dashed gray lines delimit the signal background for probes in the coding sequence and dashed red lines delimit that for antisense probes. (1) *cis*-antisense RNA to *NMB1923*; expected amplicon size, 380 bp. (2) *cis*-antisense RNA to *NMB0954*; expected amplicon size, 1,014 bp. (3) *cis*-antisense RNA to *NMB0884*; expected amplicon size, 650 bp. (4) *cis*-antisense RNA to *NMB1686*; expected amplicon size, 780 bp. (5) *cis*-antisense RNA to *NMB1786*; expected amplicon size, 240 bp. (6) *cis*-antisense RNA to *NMB1985*; expected amplicon size, 2,200 bp.

tracted at t_0 (for downregulated transcripts) or t_{60} (for upregulated transcripts) (Fig. 3B and 5).

Among all the antisense transcripts identified, some corresponded to genes potentially involved in virulence and pathogenesis. As an example, *NMB0752* is a putative bacterioferritin-associated ferredoxin strongly upregulated in blood (M value, 3.05); on the other hand, its asRNA was downregulated (M value, -1.68). This suggests that the protein could be necessary for survival in blood and regulated by a transcript that therefore needs to be downregulated. Moreover, it has been reported that ferredoxin is important for *E. coli* pathogenesis (70).

There are several other examples of upregulation of the mRNA and downregulation of the corresponding asRNA (Fig. 3B; see Table S2 in the supplemental material). In other cases, as already mentioned, both transcripts were upregulated, for example, the type II citrate synthase *NMB0954* (Fig. 5B2). Among cases where only the antisense RNA was differentially expressed, an interesting example is the downregulated *cis*-antisense transcript corresponding to the superoxide dismutase *sodB* (*NMB0884*) (Fig. 5B3). The result is consistent with the deregulation of the protein observed in Hfq deletion mutants (13, 45) and supports the idea that the expression of *sodB* is posttranscriptionally regulated, perhaps by its *cis*-encoded antisense RNA. This finding is not surprising, since Lorenz and colleagues reported the presence of Hfq-binding sites in many antisense RNAs in *E. coli*, suggesting that Hfq might regulate the expression of a large number of genes via interaction with *cis*-antisense RNAs (33). Other cases of antisense RNAs to ORFs deregulated in Hfq deletion mutants are given in the notes in Fig. 3B.

Another example is the asRNA of *lrp* (*NMB1650*) (Fig. 3B), significantly downregulated during incubation in blood. *lrp* is a leucine-binding transcriptional regulator that is not differentially expressed in blood, but the downregulation of the antisense RNA suggests instead that the gene was somehow regulated in blood. Interestingly, the regulation of Lrp by the MicF small RNA in *E. coli* has been recently reported (10, 24). Moreover, in the literature, there are other examples of antisense RNAs shown to regulate the synthesis of transcription regulators, either positively, like *gadY* of *E. coli* acting on the stability of *gadX* mRNA (43) or *arnA* of *Corynebacterium glutamicum* on *cg1935* (71), or negatively, like *alr1690* of *Anabaena* sp. on *furA* (21).

In some cases, we found antisense RNAs corresponding to genes that were part of an operon, for example, the asRNA to *NMB0432*, which seems to be cotranscribed with *NMB0433*. The same was observed for the asRNA to *putA* (*NMB0401*) and *exbB* (*NMB1729*) (Fig. 3B). Interestingly, for the operon *NMB1652*-*NMB1653*, a downregulated asRNA for *NMB1652* and an upregulated asRNA for *NMB1653* were detected (see Tables S2 and S4 in the supplemental material).

In one case, the antisense strand of the 81-bp predicted ORF *NMB0362* was flanked by two upregulated intergenic transcriptional units. RACE analysis was performed using a pair of primers designed in the intergenic region *NMB0362*-*NMB0363*, and it resulted in a longer RT-PCR product, comprising the other two probes in IG *NMB0361*-*NMB0362*. We considered this 273-nt transcript an antisense RNA of *NMB0362*, and on the basis of the sequence obtained by RACE analysis, it was also possible to determine the presence of a putative promoter and a Rho-independent terminator (see Figure S3 in the supplemental material).

Transcripts with extended 5' and 3' UTRs. Mapping of the

identified transcriptional units in the genome showed the presence of a 5' and/or 3' UTR within the transcripts of 88 genes (see Table S3 in the supplemental material). The lengths of these regions are variable and range from 60 to 531 nt. The 5' UTR is defined as the region between the transcriptional start site and the start codon of an mRNA, while a long 3' UTR can be generated either by the absence of a transcriptional terminator near the stop codon or by termination read-through events (47, 48). In total, 24 5' UTRs and 52 3' UTRs were identified; in addition, 12 transcripts comprised both a 5' and 3' UTR (Fig. 1B). Two 5' UTRs and five 3' UTRs were confirmed experimentally by performing an RT-PCR with specific pairs of primers in order to discriminate the presence of a long transcript comprising the UTR (for the PCR, we used total RNA collected at time zero for downregulated transcripts or after 60 min of incubation in blood for upregulated transcripts) (Table 1 and Fig. 6). These untranslated regions are reported to have regulatory functions and are used by pathogenic bacteria to modify the expression or translation of the mRNA itself (16, 46). 5' UTRs can be used to regulate gene expression on the basis of changes in temperature and pH and the presence of metabolites. The last group of 5' UTRs is represented by riboswitches. These are metabolite-sensing regulatory RNA structures that function as sensors and regulators of various metabolic pathways in bacteria and can be classified according to the metabolite they bind (20, 42). In our analysis, 16 5' UTRs could potentially have this regulatory activity (see Table S3 in the supplemental material), but using Rfam, no known function was assigned. However, a 253-nt 5' UTR was identified in the mRNA of *leuA* (*NMB1070*) (Table 1). *LeuA* is a 2-isopropylmalate synthase involved in leucine biosynthesis, and future experiments could classify its 5' UTR as a new riboswitch, able to regulate the expression of the gene according to the availability of leucine (as reported for other amino acids, i.e., glycine and lysine). Interestingly, a similar mechanism has been proposed for the 5' UTR of leucyl-tRNA synthetase (named Teg73) of *Staphylococcus aureus* (2). The same could be true of the 210-nt 5' UTR of *amtB* (*NMB0615*) (Fig. 6B), which encodes a putative ammonium transporter.

On the other hand, the 3' UTRs of bacterial mRNAs are thought to mainly harbor transcription termination structures, which might prevent access of exonucleases to the 3' end of the transcript. These long putative 3' regulatory UTRs have been observed next to transcripts encoding certain virulence factors in *S. aureus*. In particular, in this bacterium, long 3' UTRs are processed and give rise to different sRNAs (2). In our analysis of the *N. meningitidis* transcriptome, several genes, differentially expressed in blood, possessed a 3' UTR. Two of them, the *NMB1712* and *NMB0983* 3' UTRs, were confirmed by RT-PCR (Fig. 6C). Interestingly, the 3' UTR of *NMB0983* seemed also to be present in our sample as a small RNA; in fact, the RACE analysis, performed using a pair of primers designed in the sequence corresponding to the 3' UTR, led to an RT-PCR product of 236 bp (see Figure S4 in the supplemental material), called Bns7, that could derive from an independent promoter or from processing of the longer transcript.

In 21 cases, the transcripts ended inside or after an adjacent gene located on the opposite strand. Thus, the transcript extended over the convergent gene, leading to an overlapping UTR (overall, 18 overlapping 3' UTRs and 3 overlapping 5' UTRs were identified). The transcription of 3 long 3' UTRs was confirmed experimentally by RT-PCR (Table 1 and Fig. 6D). How these overlap-

TABLE 1 Transcripts with extended 5' and 3' UTRs confirmed by RT-PCR^a

Strand	ORF	Annotation/function	Start site	End site	Overlap	UTR	Length (nt)	M value (log ₂ Cy5/Cy3)			
								t ₀	t ₃₀	t ₆₀	t ₉₀
Downregulated genes											
F	NMB1452	Conserved hypothetical protein	1500497	1503255	NMB1453-NMB1454	3′	1,617	−0.005	−2.608	−2.847	−2.829
Upregulated genes											
R	NMB0615	Putative ammonium transporter; AmtB	645065	646584		5′	210	0.050	2.580	3.456	3.451
F	NMB0983	Purine ribonucleotide biosynthesis; PurH	998362	1000469		3′	531	0.095	2.740	2.202	2.004
F	NMB1070	2-Isopropylmalate synthase; LeuA	1090259	1092068		5′	253	0.106	1.026	1.475	1.566
R	NMB1647	Putative amino acid symporter	1713234	1715531	NMB1646	3′	791	0.107	2.306	1.765	1.940
F	NMB1710	Glutamate dehydrogenase	1786070	1787743	NMB1711	3′	345	0.039	2.816	2.696	2.681
F	NMB1712	L-Lactate permease-related protein	1788748	1789214		3′	168	0.079	2.519	3.129	2.935
F	NMB1840	Conserved hypothetical protein	1941540	1942748	NMB1841	3′	701	0.014	0.872	1.860	2.124

^a A subpanel of *N. meningitidis* transcripts containing an extended UTR at the 5' or 3' end that were confirmed by RT-PCR is described. For each transcript, the following are defined: the strand (forward [F] for a 5'-3' genomic orientation and reverse [R] for the opposite strand); the ORF of the transcript; the start and end of the transcriptional unit; the length of the UTR; and the transcriptional profile of the transcript during the time course experiment, reported as the *M* value (the log₂ ratio between the two channels, Cy5 for 30, 60, and 90 min of incubation in blood and Cy3 for the reference condition at *t*₀).

ping UTRs affect gene expression is not clearly understood, but it has been reported that they can act as antisense RNAs for the gene located in the opposite strand (64). This could also be a way to link the expression of neighboring genes in the context of global gene regulation. In our cases, the *NMB1710* long 3' UTR overlapped with the downstream ORF *NMB1711*. *NMB1710* codes for the glutamate dehydrogenase *gdhA*, while *NMB1711* belongs to the *gntR* family of transcriptional regulators. This suggests that the long 3' UTR, which is antisense to *NMB1711*, is used as a connection between two metabolic pathways. Another example is the 3' UTR of *NMB1840* (coding for a hypothetical protein) that overlapped for 701 nt with *NMB1841*. It is worth noting that *NMB1841* seemed to be coexpressed with *NMB1842* as a putative operon (see Table S4 in the supplemental material), and they code, respectively, for a mannose-1-phosphate guanylyltransferase and a putative 4-hydroxyphenylacetate 3-hydroxylase involved in different metabolic pathways.

In the context of adaptation to blood, it is also worth mentioning the *NMB1452* 3' UTR (Table 1). This long 3' UTR of 1,617 nt was strongly downregulated (*M* value, −2.8 at *t*₆₀) and overlapped with *NMB1453* and *NMB1454*. *NMB1454* is a ferredoxin and was not differentially expressed in blood. On the other hand, as mentioned above, another putative bacterioferritin-associated ferredoxin (*NMB0752*) was upregulated while its antisense was downregulated. This suggests finely tuned regulation of this system in *N. meningitidis*.

Differentially expressed operons. The gene expression data obtained from probes designed in the coding sequence of the genes were used to define the operon map of differentially expressed genes during incubation in human blood. A total of 376 transcriptional units overlapped with 624 annotated ORFs. Of these, 141 units overlapped with two or more ORFs, suggesting a possible operon with polycistronic transcription of the locus. In detail, the identification process started with a list of 376 transcriptional units, determined using the chipSAD software, and the fol-

lowing rules were applied: (i) *M* values for all the probes designed in the coding sequence of adjacent genes were similar; (ii) *M* values for the probes designed in the intergenic regions between adjacent genes and the probes designed inside the genes, when present, were similar; and (iii) an adequate statistical significance of the probes belonging to the transcriptional unit. The analysis resulted in the identification of 141 regulated operons; nearly 63% consisted of two genes, while in some cases a single transcriptional unit contained more than three genes clustered together (Fig. 1C; see Table S4 in the supplemental material). The presence in our samples of long regulated transcripts, corresponding to operons, was confirmed by RT-PCR with specific primers, in order to amplify both the entire operon and the intergenic regions between adjacent genes, using primers designed inside the genes in divergent orientations (Fig. 7A). As an example, the three genes *NMB0429*, *NMB0430*, and *NMB0431* were part of the same transcriptional unit containing both probes inside the genes and probes in the intergenic regions (Fig. 7B). *NMB0429* is annotated as encoding a small hypothetical protein of 34 amino acids (aa), while *NMB0430* and *NMB0431* code for PrpB (2-methylisocitrate lyase) and PrpC (methylcitrate synthase), respectively, and were all strongly upregulated in blood. Interestingly, the *NMB0429* sequence is the best hit as the target mRNA for Bns1. Moreover, an antisense transcript for *prpB* and *prpC* was detected (Fig. 3B), and the PrpB and PrpC proteins were deregulated in an Hfq deletion mutant (13, 36, 45).

Another case is the transcriptional unit that includes the genes *NMB1728*, *NMB1729*, and *NMB1730*. The genes code for the biopolymer proteins ExbD and ExbB and the TonB protein, respectively, and are involved in cation uptake. This system is required for heme utilization and virulence in various bacteria (22, 56, 60). The analysis showed the upregulation of the operon during incubation in human blood; in addition, an upregulated antisense transcript to *NMB1729* was detected (Fig. 3B). Moreover, *NMB1730* was identified as a possible target sequence for Bns4. All

FIG 6 Detection of identified transcripts with extended 5' and 3' UTRs by RT-PCR. (A) Scheme of the RT-PCR strategy used to confirm the presence of 5' and 3' untranslated regions in RNA transcripts extracted after incubation of *N. meningitidis* in human whole blood. We used two pairs of primers, one to amplify only the coding sequence as a positive control (1) and one to amplify the entire transcript (corresponding to the signal obtained from the tiling array) in order to confirm the presence of a 5' or 3' untranslated region (2). The dashed blue arrow indicates the mRNA. In red arrows are the primers used

these findings suggested finely tuned regulation of the expression of this system during incubation in human blood.

Because functionally related genes are generally clustered in operons, identification of operons is critical for gene function elucidation. In this regard, in some cases, the identified transcriptional units corresponded to an annotated ORF clustered with genes encoding hypothetical proteins, for example, *NMB0432-acnA*, *folB-NMB1064*, and *leuD-NMB1035* (see Table S4 in the supplemental material). This analysis suggests not only that these genes are somehow involved in growth and survival in human blood, but also that their activities could be correlated. The same reasoning can be applied to adjacent genes with known functions, such as the long transcriptional unit that includes the putative oxalate/formate antiporter *NMB1362* and the exodeoxyribonuclease VII large subunit *eseA* (*NMB1363*), shown in Fig. 7C.

Interestingly, in 7 cases, the transcriptional unit extended to an adjacent gene mapped in the opposite strand, possibly acting as an antisense RNA to that gene. Two examples are *NMB0787-NMB0788-NMB0789*, which has a long 3' UTR overlapping with *NMB0790* (Fig. 7D), and *cstA-NMB1494*, which has a long 5' UTR overlapping with *NMB1492* (see Table S4 in the supplemental material).

We checked the correspondence of the operons identified with a list of predicted *N. meningitidis* MC58 operons available in the DOOR database (34). The analysis showed that, using our approach, we were able to identify 31 transcriptional units not predicted by the DOOR algorithm, while for 49 transcriptional units, the operon organization was different; the remaining 61 transcripts corresponded.

It is important to emphasize that the fact that we found the same signal in adjacent genes (not previously predicted to be part of an operon) means that they are transcriptionally correlated, but when there is not a probe designed in the intergenic region between two genes, we can only speculate that they are part of the same operon. Further experiments will be necessary to confirm these findings.

DISCUSSION

One of the crucial steps in bacterial pathogenesis is adaptation to the host environment and different host factors; the ability of *N. meningitidis* to survive and multiply in the host is mainly due to its capacity to regulate gene expression. *N. meningitidis* is an exclusively human pathogen, and existing animal models may not accurately simulate meningococcal disease. This justifies the use of an experimental system that mimics, as closely as possible, the *in vivo* situation seen during disease. In this work, the total neisserial transcriptome was analyzed in a time course experiment of incubation in an *ex vivo* model of bacteremia, using a tiling array. The *ex vivo* human whole-blood model has shown potential to examine a number of parameters that are likely to be important in the cascade of events associated with acute systemic meningococcal

for RT-PCR; the light red arrows indicate the primers used for PCR1 and -2. (B to D) Examples of a 5' UTR (*NMB0615* [*amtB*]; expected amplicon sizes, 1,100 bp [PCR1] and 1,350 bp [PCR2]) (B), a 3' UTR (*NMB1712*; expected amplicon sizes, 187 bp [PCR1] and 463 bp [PCR2]) (C), and overlapping 3' UTRs (*NMB1840*; expected amplicon sizes, 620 bp [PCR1] and 1,070 bp [PCR2]) (D). For each example, the transcriptional map (a) and the results of RT-PCR (b) are shown.

FIG 7 Detection of differentially expressed operons by RT-PCR. (A) Scheme of the RT-PCR strategy used to confirm the presence of long transcripts, referred to as operons, in total RNA extracted after incubation of *N. meningitidis* in human whole blood. Reverse transcription was performed using a primer designed at the end of the detected transcript. Then, PCR analysis was performed using the same cDNA sample in order to amplify the entire transcript (PCR1) and the intergenic regions between adjacent genes (PCR2 and PCR3). The dashed blue arrow indicates the entire mRNA. In light red arrows are the primers used for RT-PCR of the entire transcript; in dark red arrows are the primers for the intergenic regions. The expected PCR products are indicated by light blue lines. (B and C) Examples of the detected operons. For each example, the transcriptional map (a) and the results of RT-PCR (b) are shown. (B) Long transcript for *NMB0429-NMB0430-NMB0431* with expected amplicon sizes: PCR1, 2,330 bp; PCR2, 300 bp; PCR3 256 bp. (C) Long transcript for *NMB1362-NMB1363* with expected amplicon sizes: PCR1, 3,000 bp; PCR2, 414 bp. (D) Long transcript for *NMB0787-NMB0788-NMB0789* with expected amplicon size: PCR1, 2,700 bp. In this case, since there are no intergenic spaces between the genes, only one PCR was performed, and it allowed the detection of a long transcript that partially overlapped *NMB0790* in the opposite strand.

infection (26) and to characterize *N. meningitidis* factors involved in the survival of the bacterium during infection (13, 52).

The application of the chipSAD tool to tiling array data allowed the identification of new transcripts—small intergenic RNAs, *cis*-encoded antisense RNAs, mRNAs with extended 5' and 3' UTRs, and operons—differentially expressed in human blood.

These findings extend our knowledge of the whole transcriptome of *N. meningitidis*; previously, other research groups had shown the expression in *N. meningitidis* of small regulatory RNAs with a role in bacterial pathogenesis (14, 35, 38), and proteomic and transcriptomic analysis of Hfq deletion mutants suggested the presence of a small-RNA network in the bacterium (13, 36, 45). Here, we reported a panel of expressed small RNAs that could be involved in the adaptation of *N. meningitidis* to the human blood environment. In fact, some sRNAs that could potentially regulate genes involved in bacterial metabolism (such as Bns1 for the methylcitrate synthesis pathway) and transport and binding (such as Bns4 for the regulation of the *tonB* gene) were significantly regulated during incubation in human blood. Moreover, we identified an sRNA, Bns3, transcribed from a prophage DNA element that can potentially regulate bacterial genes, as was already described for the *sprD* sRNA of *S. aureus* (6) and *dicF* (4) and *ipeX* (5) of *E. coli* and that might support the idea of the involvement of bacteriophages in bacterial pathogenesis (3, 63).

In this work, we showed, for the first time in *N. meningitidis*, extensive antisense transcription when bacteria were incubated in human whole blood. This means not only that *N. meningitidis* might also use antisense transcription to regulate gene expression, but that these riboregulators contribute to adaptation of the bacteria to grow and survive in human blood. In fact, antisense RNAs to genes of various metabolic pathways were identified; some others corresponded to genes potentially involved in neisserial pathogenesis (for example, the asRNA for *app*, the ferredoxin gene *NMB0752*, and the citrate synthase gene *NMB0954*). The time course analysis allowed us to monitor changes in the transcription profile over 90 min of incubation in human blood. This study showed different types of correlation between the expression of an asRNA and that of the corresponding mRNA. In about 44.8% of cases, only the asRNA was differentially expressed. This might mean that the corresponding genes, although not regulated in our analysis, are indeed somehow involved in the adaptation to human blood. This suggests that there could be more genes than the ones already described (12, 18) implicated in this step of pathogenesis that are worthy of further investigation (59).

These different types of regulation could be explained according to the potential mechanism of action of antisense RNAs; in the majority of cases, antisense RNA action entails posttranscriptional inhibition of target RNA function, but in a few cases, activating mechanisms have also been found (43). As a general statement, if antisense transcripts were acting as negative *cis*-regulatory elements, the signal levels of the sense and antisense ratio would be expected to be inversely correlated (48). On the other hand, it is not trivial to assign a functional correlation when both sense and antisense RNAs are regulated in the same way or only the antisense RNA is differentially expressed, as observed in most cases during incubation in blood. It has been proposed that the expression of a target RNA is repressed as long as the transcription of the regulator exceeds the transcription of the target, and its expression increases linearly when the mRNA concentration outruns that of the regulator; only an mRNA that is present at higher levels than its

cis-acting antisense antagonist is translated, so translation of the ORF starts only when the mRNA concentration reaches a certain level (32). In the cases of asRNAs corresponding to genes that are part of an operon, asRNAs can be utilized by bacteria to set temporal gene expression thresholds for genes within an operon and to implement a quantitative adjustment of the mRNA amounts (31, 43, 58, 65).

According to the analysis described in this work, the RNA regulatory circuit in *N. meningitidis* appears very complex. In fact, the expression of some genes was regulated at different levels; they might be coexpressed with the adjacent gene or have a *cis*-acting antisense RNA, and the transcript can be also the target of a small RNA. As an example, the three genes *NMB0429-NMB0430-NMB0431* were cotranscribed as part of a single operon involved in the methylcitrate synthesis pathway. The sequence of *NMB0429* is a putative target of Bns1 sRNA, and an antisense transcript for *prpB* and *prpC* was detected. All these findings suggest that *N. meningitidis* needs to regulate, in diverse manners, the expression level of this operon under a particular growing condition, like human blood. Interestingly, in *M. tuberculosis*, this metabolic pathway is required for growth on fatty acids, in macrophages, and in mice (40, 66).

In conclusion, this study gives a global view of how *N. meningitidis* regulates gene expression to be able to survive and multiply in human blood as an important step in bacterial pathogenesis. Further experiments are needed to confirm a role for these new RNAs in the adaptation of meningococci to human blood, and they will also help in the study of the regulation of genes encoding important virulence factors.

ACKNOWLEDGMENTS

We thank Isabel Delany and Mariagrazia Pizza for useful discussion and critical reading of the manuscript. We also thank Hebert Echenique-Rivera and Laura Fantappiè for technical suggestions.

E.D.T. was the recipient of a Novartis fellowship from the Ph.D. Program in Functional Biology of Molecular and Cellular Systems of the University of Bologna. S.B. is the recipient of a Novartis fellowship from the Ph.D. Program in Mathematical Logic, Computer Science, and Bioinformatics of the University of Siena.

REFERENCES

1. Ansong C, et al. 2009. Global systems-level analysis of Hfq and SmpB deletion mutants in *Salmonella*: implications for virulence and global protein translation. *PLoS One* 4:e4809. doi:10.1371/journal.pone.0004809.
2. Beaume M, et al. 2010. Cartography of methicillin-resistant *S. aureus* transcripts: detection, orientation and temporal expression during growth phase and stress conditions. *PLoS One* 5:e10725. doi:10.1371/journal.pone.0010725.
3. Bille E, et al. 2008. Association of a bacteriophage with meningococcal disease in young adults. *PLoS One* 3:e3885. doi:10.1371/journal.pone.0003885.
4. Bouche F, Bouche JP. 1989. Genetic evidence that DcF, a second division inhibitor encoded by the *Escherichia coli* *dicB* operon, is probably RNA. *Mol. Microbiol.* 3:991–994.
5. Castillo-Keller M, Vuong P, Misra R. 2006. Novel mechanism of *Escherichia coli* porin regulation. *J. Bacteriol.* 188:576–586.
6. Chabelskaya S, Gaillot O, Felden B. 2010. A *Staphylococcus aureus* small RNA is required for bacterial virulence and regulates the expression of an immune-evasion molecule. *PLoS Pathog.* 6:e1000927. doi:10.1371/journal.ppat.1000927.
7. Claus H, Vogel U, Swiderek H, Frosch M, Schoen C. 2007. Microarray analyses of meningococcal genome composition and gene regulation: a review of the recent literature. *FEMS Microbiol. Rev.* 31:43–51.
8. Cummings M, McGurk C, Masters JR. 2003. Rapid identification of antisense mRNA-expressing clones using strand-specific RT-PCR. *Antisense Nucleic Acid Drug Dev.* 13:115–117.
9. Darton T, et al. 2009. Severity of meningococcal disease associated with genomic bacterial load. *Clin. Infect. Dis.* 48:587–594.
10. Delihans N. 2012. Regulating the regulator: MicF RNA controls expression of the global regulator Lrp. *Mol. Microbiol.* 84:401–404.
11. Dunning Hotopp JC, et al. 2006. Comparative genomics of *Neisseria meningitidis*: core genome, islands of horizontal transfer and pathogen-specific genes. *Microbiology* 152:3733–3749.
12. Echenique-Rivera H, et al. 2011. Transcriptome analysis of *Neisseria meningitidis* in human whole blood and mutagenesis studies identify virulence factors involved in blood survival. *PLoS Pathog.* 7:e1002027. doi:10.1371/journal.ppat.1002027.
13. Fantappiè L, et al. 2009. The RNA chaperone Hfq is involved in stress response and virulence in *Neisseria meningitidis* and is a pleiotropic regulator of protein expression. *Infect. Immun.* 77:1842–1853.
14. Fantappiè L, et al. 2011. A novel Hfq-dependent sRNA that is under FNR control and is synthesized in oxygen limitation in *Neisseria meningitidis*. *Mol. Microbiol.* 80:507–523.
15. Graham MR, et al. 2005. Group A *Streptococcus* transcriptome dynamics during growth in human blood reveals bacterial adaptive and survival strategies. *Am. J. Pathol.* 166:455–465.
16. Gripenland J, et al. 2010. RNAs: regulators of bacterial virulence. *Nat. Rev. Microbiol.* 8:857–866.
17. Hackett SJ, et al. 2002. Meningococcal bacterial DNA load at presentation correlates with disease severity. *Arch. Dis. Child.* 86:44–46.
18. Hedman AK, Li MS, Langford PR, Kroll JS. 2012. Transcriptional profiling of serogroup B *Neisseria meningitidis* growing in human blood: an approach to vaccine antigen discovery. *PLoS One* 7:e39718. doi:10.1371/journal.pone.0039718.
19. Hellerud BC, et al. 2008. Stages of meningococcal sepsis simulated in vitro, with emphasis on complement and Toll-like receptor activation. *Infect. Immun.* 76:4183–4189.
20. Henkin TM. 2008. Riboswitch RNAs: using RNA to sense cellular metabolism. *Genes Dev.* 22:3383–3390.
21. Hernandez JA, et al. 2006. Identification of a furA *cis* antisense RNA in the cyanobacterium *Anabaena* sp. PCC 7120. *J. Mol. Biol.* 355:325–334.
22. Holden KM, Browning GF, Noormohammadi AH, Markham PF, Maranda MS. 2012. TonB is essential for virulence in avian pathogenic *Escherichia coli*. *Comp. Immunol. Microbiol. Infect. Dis.* 35:129–138.
23. Hollander M, Wolfe DA. 1999. Nonparametric statistical methods, 2nd ed. Wiley, New York, NY.
24. Holmqvist E, Unoson C, Reimegard J, Wagner EG. 2012. A mixed double negative feedback loop between the sRNA MicF and the global regulator Lrp. *Mol. Microbiol.* 84:414–427.
25. Huber W, Toedling J, Steinmetz LM. 2006. Transcript mapping with high-density oligonucleotide tiling arrays. *Bioinformatics* 22:1963–1970.
26. Ison CA. 2001. Whole-blood model. *Methods Mol. Med.* 66:317–329.
27. Ison CA, Heyderman RS, Klein NJ, Peakman M, Levin M. 1995. Whole blood model of meningococcal bacteraemia—a method for exploring host-bacterial interactions. *Microb. Pathog.* 18:97–107.
28. Jonnalagadda S, Srinivasan R. 2008. Principal components analysis based methodology to identify differentially expressed genes in time-course microarray data. *BMC Bioinformatics* 9:267.
29. Julio SM, Heithoff DM, Mahan MJ. 2000. *ssrA* (tmRNA) plays a role in *Salmonella enterica* serovar Typhimurium pathogenesis. *J. Bacteriol.* 182:1558–1563.
30. Kawano M, Aravind L, Storz G. 2007. An antisense RNA controls synthesis of an SOS-induced toxin evolved from an antitoxin. *Mol. Microbiol.* 64:738–754.
31. Lee EJ, Groisman EA. 2010. An antisense RNA that governs the expression kinetics of a multifunctional virulence gene. *Mol. Microbiol.* 76:1020–1033.
32. Levine E, Zhang Z, Kuhlman T, Hwa T. 2007. Quantitative characteristics of gene regulation by small RNA. *PLoS Biol.* 5:e229. doi:10.1371/journal.pbio.0050229.
33. Lorenz C, et al. 2010. Genomic SELEX for Hfq-binding RNAs identifies genomic aptamers predominantly in antisense transcripts. *Nucleic Acids Res.* 38:3794–3808.
34. Mao F, Dam P, Chou J, Olman V, Xu Y. 2009. DOOR: a database for prokaryotic operons. *Nucleic Acids Res.* 37:D459–D463.
35. Mellin JR, Goswami S, Grogan S, Tjaden B, Genco CA. 2007. A novel fur- and iron-regulated small RNA, NrrF, is required for indirect fur-mediated regulation of the *sdhA* and *sdhC* genes in *Neisseria meningitidis*. *J. Bacteriol.* 189:3686–3694.

36. Mellin JR, et al. 2010. Role of Hfq in iron-dependent and -independent gene regulation in *Neisseria meningitidis*. *Microbiology* 156:2316–2326.
37. Mereghetti L, Sitkiewicz I, Green NM, Musser JM. 2008. Extensive adaptive changes occur in the transcriptome of *Streptococcus agalactiae* (group B streptococcus) in response to incubation with human blood. *PLoS One* 3:e3143. doi:10.1371/journal.pone.0003143.
38. Metruccio MM, et al. 2009. The Hfq-dependent small noncoding RNA NrrF directly mediates Fur-dependent positive regulation of succinate dehydrogenase in *Neisseria meningitidis*. *J. Bacteriol.* 191:1330–1342.
39. Mollnes TE, et al. 2002. Essential role of the C5a receptor in *E. coli*-induced oxidative burst and phagocytosis revealed by a novel lepirudin-based human whole blood model of inflammation. *Blood* 100:1869–1877.
40. Munoz-Elias EJ, Upton AM, Cherian J, McKinney JD. 2006. Role of the methylcitrate cycle in *Mycobacterium tuberculosis* metabolism, intracellular growth, and virulence. *Mol. Microbiol.* 60:1109–1122.
41. Nolte O, Rickert A, Ehrhard I, Ledig S, Sonntag HG. 2002. A modified ex vivo human whole blood model of infection for studying the pathogenesis of *Neisseria meningitidis* during septicemia. *FEMS Immunol. Med. Microbiol.* 32:91–95.
42. Nudler E, Mironov AS. 2004. The riboswitch control of bacterial metabolism. *Trends Biochem. Sci.* 29:11–17.
43. Opdyke JA, Kang JG, Storz G. 2004. GadY, a small-RNA regulator of acid response genes in *Escherichia coli*. *J. Bacteriol.* 186:6698–6705.
44. Ovstebo R, et al. 2004. Use of robotized DNA isolation and real-time PCR to quantify and identify close correlation between levels of *Neisseria meningitidis* DNA and lipopolysaccharides in plasma and cerebrospinal fluid from patients with systemic meningococcal disease. *J. Clin. Microbiol.* 42:2980–2987.
45. Pannekoek Y, RHuis in 't Veld Hopman CT, Langerak AA, Speijer D, van der Ende A. 2009. Molecular characterization and identification of proteins regulated by Hfq in *Neisseria meningitidis*. *FEMS Microbiol. Lett.* 294:216–224.
46. Papenfort K, Vogel J. 2010. Regulatory RNA in bacterial pathogens. *Cell Host Microbe* 8:116–127.
47. Peters JM, et al. 2009. Rho directs widespread termination of intragenic and stable RNA transcription. *Proc. Natl. Acad. Sci. U. S. A.* 106:15406–15411.
48. Rasmussen S, Nielsen HB, Jarmer H. 2009. The transcriptionally active regions in the genome of *Bacillus subtilis*. *Mol. Microbiol.* 73:1043–1057.
49. Royce TE, Carriero NJ, Gerstein MB. 2007. An efficient pseudomedian filter for tiling microarrays. *BMC Bioinform.* 8:186.
50. Saeed AI, et al. 2003. TM4: a free, open-source system for microarray data management and analysis. *Biotechniques* 34:374–378.
51. Schwerk C, et al. 2012. Polar invasion and translocation of *Neisseria meningitidis* and *Streptococcus suis* in a novel human model of the blood-cerebrospinal fluid barrier. *PLoS One* 7:e30069. doi:10.1371/journal.pone.0030069.
52. Seib KL, et al. 2009. Factor H-binding protein is important for meningococcal survival in human whole blood and serum and in the presence of the antimicrobial peptide LL-37. *Infect. Immun.* 77:292–299.
53. Sharma CM, et al. 2010. The primary transcriptome of the major human pathogen *Helicobacter pylori*. *Nature* 464:250–255.
54. Song T, et al. 2008. A new *Vibrio cholerae* sRNA modulates colonization and affects release of outer membrane vesicles. *Mol. Microbiol.* 70:100–111.
55. Sprong T, et al. 2003. Inhibition of C5a-induced inflammation with preserved C5b-9-mediated bactericidal activity in a human whole blood model of meningococcal sepsis. *Blood* 102:3702–3710.
56. Stojiljkovic I, Srinivasan N. 1997. *Neisseria meningitidis* tonB, exbB, and exbD genes: Ton-dependent utilization of protein-bound iron in *neisseriae*. *J. Bacteriol.* 179:805–812.
57. Storey JD, Tibshirani R. 2003. Statistical significance for genomewide studies. *Proc. Natl. Acad. Sci. U. S. A.* 100:9440–9445.
58. Stork M, Di Lorenzo M, Welch TJ, Crosa JH. 2007. Transcription termination within the iron transport-biosynthesis operon of *Vibrio anguillarum* requires an antisense RNA. *J. Bacteriol.* 189:3479–3488.
59. Tang F, Lao K, Surani MA. 2011. Development and applications of single-cell transcriptome analysis. *Nat. Methods* 8:S6–S11.
60. Tauseef I, et al. 2011. Influence of the combination and phase variation status of the haemoglobin receptors HmbR and HpuAB on meningococcal virulence. *Microbiology* 157:1446–1456.
61. Tettelin H, et al. 2000. Complete genome sequence of *Neisseria meningitidis* serogroup B strain MC58. *Science* 287:1809–1815.
62. Thomassen GO, Rowe AD, Lagesen K, Lindvall JM, Rognes T. 2009. Custom design and analysis of high-density oligonucleotide bacterial tiling microarrays. *PLoS One* 4:e5943. doi:10.1371/journal.pone.0005943.
63. Tinsley CR, Bille E, Nassif X. 2006. Bacteriophages and pathogenicity: more than just providing a toxin? *Microbes Infect.* 8:1365–1371.
64. Toledo-Arana A, et al. 2009. The *Listeria* transcriptional landscape from saprophytism to virulence. *Nature* 459:950–956.
65. Tramonti A, De Canio M, De Biase D. 2008. GadX/GadW-dependent regulation of the *Escherichia coli* acid fitness island: transcriptional control at the gadY-gadW divergent promoters and identification of four novel 42 bp GadX/GadW-specific binding sites. *Mol. Microbiol.* 70:965–982.
66. Upton AM, McKinney JD. 2007. Role of the methylcitrate cycle in propionate metabolism and detoxification in *Mycobacterium smegmatis*. *Microbiology* 153:3973–3982.
67. Virji M. 2009. Pathogenic *neisseriae*: surface modulation, pathogenesis and infection control. *Nat. Rev. Microbiol.* 7:274–286.
68. Vogel J, Luisi BF. 2011. Hfq and its constellation of RNA. *Nat. Rev. Microbiol.* 9:578–589.
69. Welsch JA, Granoff D. 2007. Immunity to *Neisseria meningitidis* group B in adults despite lack of serum bactericidal antibody. *Clin. Vaccine Immunol.* 14:1596–1602.
70. Yu H, Kim KS. 2010. Ferredoxin is involved in secretion of cytotoxic necrotizing factor 1 across the cytoplasmic membrane in *Escherichia coli* K1. *Infect. Immun.* 78:838–844.
71. Zemanova M, et al. 2008. Chromosomally encoded small antisense RNA in *Corynebacterium glutamicum*. *FEMS Microbiol. Lett.* 279:195–201.