Production of the signalling molecule, autoinducer-2, by Neisseria meningitidis: lack of evidence for a concerted transcriptional response

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INTRODUCTION

Bacteria, as single-celled prokaryotic organisms, were originally thought not to exhibit coordinated, organized behaviour. It is now known, however, that they have complex communication systems that allow bacterial communities to react to changes in growth conditions, most often in response to population density, a process called quorum sensing (de Kievit & Iglewski, 2000; Schauder & Bassler, 2001; Whitehead et al., 2001). In systems studied to date, cell-to-cell communication relies on the production and release of small signalling molecules called autoinducers. Once an extracellular threshold concentration of the molecule has been reached, corresponding to a specific density of bacteria, a set of genes are co-ordinately activated or repressed within the bacterial population. The best characterized quorum sensing systems involve the production of species-specific signalling molecules, N-acyl homoserine lactones for Gram-negative bacteria and small post-translationally processed peptides for Gram-positive bacteria. A wide range of processes involved in symbiosis and pathogenesis are regulated by quorum sensing, including biofilm formation by Pseudomonas aeruginosa (Davies et al., 1998), competence and sporulation in Bacillus subtilis (Grossman, 1995), and expression of virulence factors by Staphylococcus aureus (Ji et al., 1995) and Vibrio cholerae (Miller et al., 2002).

Vibrio harveyi has recently been shown to utilize not only a homoserine lactone signalling molecule (AI-1), but also a novel autoinducer (AI-2) to control the density-dependent induction of bioluminescence (Bassler et al., 1993, 1994). Surprisingly, an identical AI-2 molecule is believed to be produced by a number of Gram-positive and Gram-negative bacteria, suggesting that AI-2 facilitates interspecies communication among bacteria (Bassler, 1999; Schauder & Bassler, 2001). In all cases studied, the highly conserved gene, luxS, has been shown to be essential for the production of AI-2 (Bassler et al., 1997). Analysis of databases has revealed the presence of luxS homologues in over 30 bacterial species, suggesting that interspecies communication may be widespread (Schauder & Bassler, 2001). AI-2 has been demonstrated to regulate virulence factor expression in both Gram-negative and Gram-positive bacteria. In enterohaemorrhagic and enteropathogenic Escherichia coli (EHEC and EPEC), this quorum sensing system is involved in regulating the expression of type III secretion systems, as well as inducing global expression changes, including cell division, flagellar biosynthesis, chemotaxis and cell surface architecture genes (DeLisa et al., 2001; Sperandio et al., 2001). In Streptococcus pyogenes, AI-2 has been shown...
to regulate the expression of two secreted virulence factors and to modulate growth rate (Lyon et al., 2001). Although other bacteria, including Helicobacter pylori (Forsyth & Cover, 2000; Joyce et al., 2000) and Salmonella typhimurium (Beeston & Surette, 2002; Surette & Bassler, 1998), have been shown to produce AI-2, the physiological role of the signalling in these bacteria has not been determined.

In V. harveyi, detection of the AI-2 molecule has been proposed to be mediated initially by LuxP, a homologue of the soluble, periplasmic ribose-binding proteins of E. coli and Sal. typhimurium, which, in complex with AI-2, can interact with the two-component hybrid sensor kinase/response regulator protein LuxQ (Bassler et al., 1993, 1994). Through a phosphorelay, this signal results in production of luminescence in the presence of AI-2. In E. coli, a two-component regulatory system has been shown to be responsible for a portion of the transcriptional changes in response to AI-2. Similarly to V. harveyi, AI-2 in complex with the small ribose-binding protein (the E. coli LuxP homologue) is believed to interact with the sensor kinase (Sperandio et al., 2002). In Sal. typhimurium, a homologue of LuxP is believed to be involved in binding AI-2, but in this case, the signalling molecule is internalized by an ABC transporter complex (Taga et al., 2001). Thus, in Gram-negative bacteria, the requirement for a LuxP homologue to bind AI-2 has been relatively well established. In Gram-positive bacteria, the detection system for AI-2 has not been elucidated, but it is worth noting that Str. pyogenes does not appear to contain a LuxP homologue, so a different mechanism of detection may be required. Surprisingly, in some Gram-negative bacteria that produce AI-2, including H. pylori, the complete genome sequences do not reveal any LuxP homologues. This suggests that if an AI-2 detection system exists in these bacteria, it does not follow the model of V. harveyi, E. coli and Sal. typhimurium.

Neisseria meningitidis is a Gram-negative bacterium which asymptomatically colonizes between 5 and 40 % of the human population (Sim et al., 2000), but which paradoxically is also the agent of cerebrospinal meningitis, a devastating disease with a morbidity of 1–3 per 100 000 in North America and Europe and considerably higher in poorer countries. A recent report investigating the role of luxS in a serogroup B strain of N. meningitidis showed that a luxS mutant was attenuated in an infant rat model of infection for bacteraemia (Winzer et al., 2002b). In a competitive infection, injecting a mixture of the wild-type and mutant bacteria, the luxS mutants were less capable of survival and growth in the bloodstream. However, the mechanism of this attenuation is unknown and no luxP homologue was found in a search of the chromosomal sequence of N. meningitidis. This prompted us to examine the role of the AI-2 quorum sensing system in more detail. In this study, using microarray analysis of the modulation of gene expression in response to AI-2, we demonstrate that this molecule has no significant effect in N. meningitidis. This is a dramatic contrast to the global regulatory effect observed in E. coli (DeLisa et al., 2001; Sperandio et al., 2001). One possibility is that, for the meningococcus, AI-2 is a metabolic product with no signalling role (Winzer et al., 2002a). However given that N. meningitidis colonizes the nasopharynx, a niche shared with many other bacteria, a hypothesis is proposed, consistent with the absence of a LuxP homologue, that AI-2 is produced by N. meningitidis primarily to affect the behaviour of other bacterial species.

METHODS

Bacterial strains. N. meningitidis strain Z5463 (serogroup A, sequence type ST-4) is closely related to strain Z2491 (serogroup A, ST-4), whose chromosome has been sequenced (Parkhill et al., 2000) and which was used in the fabrication of the microarrays. N. meningitidis strain 8013-2C43 (serogroup C, ST-18) has been described previously (Nassif et al., 1993). Results reported were obtained with strain Z5463 unless otherwise indicated in the text.

Bacterial media and growth conditions. N. meningitidis were grown at 37 °C with a 5 % CO₂ atmosphere on GCB agar (Difco) plates containing Kellogg’s supplement (Kellogg et al., 1963). For liquid growth, cultures were inoculated from an overnight plate into GC-phosphate (Klee et al., 2000) with Kellogg’s supplement or RPMI (Gibco-LifeTech) supplemented with either 10 % heat-inactivated fetal calf serum or 1 % bovine serum albumin (BSA) in a gas permeable 75 cm² flask and incubated at 37 °C as described above.

Construction of the N. meningitidis luxS mutant. The luxS mutant of strain Z5463 was constructed by amplifying the gene from genomic DNA using primers luxS1 (5’-GCCCCTACTAGAC-GTTCTAAC-3’) and luxS2 (5’-GGGGTTCAACGCCTTCTAT-CC-3’). The PCR product was directly cloned into the TOPO TA cloning plasmid (Invitrogen) according to the manufacturer’s instructions. The resulting plasmid was digested with EcoN1, which cuts approximately 150 bases from the 5’ end of the gene. The luxS gene was disrupted with a spectinomycin resistance cassette flanked by neisserial uptake sequences and selected on plates containing 75 μg spectinomycin ml⁻¹ (Klee et al., 2000). The resulting plasmid was linearized and used to transform N. meningitidis and inactivate the luxS gene by homologous recombination according to established protocols. The luxS mutant of strain 2C43 was generated by transformation with the genomic DNA of the luxS mutant of Z5463. The mutation was selected for by plating on spectinomycin as above. PCR analysis and Southern blotting confirmed the disruption of luxS in both cases.

Generation of cell-free conditioned medium (CM). CM was prepared essentially as described previously (Bassler et al., 1997). Briefly, N. meningitidis and luxS mutants were grown in either GC-phosphate (as described above) or RPMI with either 10 % heat-inactivated fetal bovine serum or 1 % BSA. Cultures were inoculated from overnight plates and diluted to a starting OD₆₀₀ of 0.01 and 0.05 and grown to an OD₆₀₀ of between 0.6 and 0.8, unless specified in the text. CM was prepared by centrifugation for 5 min at 5000 r.p.m., followed by filtration through Nalgene (0.45 μm pore size) filters, and was stored at −20 °C and used within 2 weeks. No changes in activity were observed during storage.

AI-2 assay. The bioluminescence assay was performed essentially as described by Bassler et al. (1993, 1994). The V. harveyi reporter strain BB170 was grown overnight in AB medium and then diluted 1:5000 into fresh AB medium containing 10 % CM. Either 10 % sterile medium (corresponding to the growth medium used for the samples) was added for a negative control or 10 % CM from V. harveyi strain BB152 and 10 % sterile medium (because the medium was

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found to affect the luminescence of V. harveyi were added for the positive control. Luminescence was measured at different times during growth with a luminometer. Induction of luminescence was determined at the time of maximal difference between the positive and negative controls (4–5 h) and is expressed as a percentage of the induction observed with CM from wild-type V. harveyi strain BB152. Growth rates, based on c.f.u. were found to be essentially the same for all samples.

N. meningitidis infection of epithelial cells. T84 cells are a human polarized intestinal epithelial cell line. The epithelial cells were cultured and seeded for adherence assays as described previously (Pujol et al., 1999). For infection, bacteria were grown on a plate overnight from frozen stock, resuspended in infection medium (RPMI 1640 with glutamax I; Gibco-BRL) supplemented with 10% heat-inactivated fetal calf serum), and approximately 10^7 bacteria were added in a volume of 1 ml to T84 cell monolayers in 24-well plates. Following adherence for 30 min, the wells were washed vigorously three times with infection medium to remove non-adherent bacteria. The number of adherent bacteria was determined by lifting off the monolayer by scraping, then vortexing the cells to dissociate bacteria and plating dilutions to determine c.f.u. Approximately 1% of the bacteria were found to adhere under these conditions. The time course of clump formation and spreading was determined visually using a phase-contrast microscope, and the quantity of bacteria at different time points was determined as above.

Gene array construction. DNA arrays were developed in collaboration with Eurogentec (Belgium). Primers were designed according to the chromosomal sequence of strain Z2491 (http://www.sanger.ac.uk/Projects/N_meningitidis/) to amplify predicted ORFs. In the case of ORFs of over 0.75 kb, only the last 0.75 kb (3’ end) of the gene was amplified. ORFs present in the MC58 genomic sequence (Tettelin et al., 2000), but absent (having less than 10% predicted amino acid homology) from strain Z2491, were also added to the membranes. Reactions (100 μl) contained 10 ng chromosomal DNA, 0.05 μM each oligonucleotide, 0.2 μM each of the four dNTPs and 0.25 U thermostable DNA polymerase. Reaction conditions were as follows: 1 min at 95°C, 30 sec at 50°C, 1 min at 72°C (30 cycles) and 7 min at 72°C (1 cycle). PCR products were confirmed by gel electrophoresis and in some cases also by sequencing. A list of primers used in the production of the DNA arrays is available as supplementary data at http://mic.sgmjournals.org. In total 2077 (98%) of the 2121 predicted genes were also added for the synthesis of suitable PCR products are noted in the supplementary data.

Images were revealed with a STORM (Amersham Pharmacia Biotech) scanner, documented asdirected by the manufacturer’s instructions (User Bulletin No. 2; ABI PRISM 7700 Sequence Detection System). The conditions were used for each reaction. The quality of the membranes was tested by multiple reactions of the membrane with the same and different membranes with labelled chromosome from the homologous strain Z2491. Inter-spot variability for the pairs (mean variation of the spots from the mean) was less than 10% for 99% of the spots, and less than 20% for 99-9% of the spots. For inter-experiment comparisons, the standard deviation was less than 20% for 99% of the spots, and less than 30% for 99-9% of the spots.

Culture of bacteria for extraction of RNA. The effect of AI-2 on transcription was determined using the wild-type and luxS mutant of strain Z2491, which is closely related to strain Z2491 used in the microarray construction. Cultures of the luxS mutant were grown in GCB to an OD_{600} of 0.8 as described above. Cultures were split into two parallel tubes and centrifuged (2500 g for 5 min). Cells were gently resuspended to an OD_{600} of 0.8 in CM taken from cultures of the wild-type or luxS mutant (+ AI-2 and −AI-2, respectively) at an OD_{600} of 0.8, thus maintaining a constant cell density but changing the concentration of AI-2. The presence or absence of AI-2 in CM was verified by a luminescence assay with V. harveyi. Growth was continued for 30 min before harvesting the bacteria by a brief centrifugation.

For experiments testing the response of the bacteria to human serum, the bacteria were first acclimatized to low iron concentrations by 2 h growth in RPMI containing 1 μM desferrioxamine. Cultures were split into two parallel tubes, then harvested by centrifugation and resuspended to an OD_{600} of 0.1 in 25% normal human serum or in serum heated at 56°C for 30 min to inactivate the complement. Growth was continued for 6 h.

RNA isolation, labelling and hybridization. Bacteria harvested by centrifugation from liquid culture were resuspended in PBS and added to tubes at 65°C containing 700 μl Trizol reagent (Lifetech) and ~50 μl of acid-washed glass beads. The tubes were vortexed vigorously and freeze-thawed using dry ice. Chloroform (200 μl) was added and the contents were mixed by shaking followed by centrifugation at 15 000 g for 15 min at 4°C. The aqueous phase was re-extracted once, then RNA was precipitated by addition of 1 vol. 2-propanol. The tubes were centrifuged at 15 000 g for 10 min at 4°C and the RNA pellet was washed with 75% ethanol, dried and redissolved in RNase-free TE buffer.

Radionlabelled cDNA was prepared after heating the RNA to 70°C for 5 min, to destabilize the secondary structure, and rapid cooling on ice. Incorporation of [α-32P]dCTP was performed by reverse transcription for 90 min at 42°C using 2 μg RNA, 5 μg random hexamer primers and Superscript II enzyme (Lifetech) according to the manufacturer’s recommendations. Hybridization (in 0.5 M sodium phosphate, 7% SDS, 1 mM EDTA, pH 7-2) and washing (in 40 mM sodium phosphate, 1% SDS, 1 mM EDTA, pH 7-2) of the membranes was performed as described by Church & Gilbert (1984).

Real time PCR assays. Oligonucleotides were designed using Primer Express software (Perkin-Elmer Applied Biosystems) to obtain amplicons of the same size. Reverse transcription was performed using the 3′ oligonucleotides of the genes and superscript TM II reverse-transcriptase (Lifetech); 50 min at 45°C and 10 min at 70°C. Reverse transcription products were diluted to 1:10 and 1:100. Real-time PCR was run on an ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems) using SYBR Green PCR Master Mix (Perkin-Elmer Applied Biosystems) according to the manufacturer’s instructions.

Data analyses for a relative quantification of gene expression were performed by the comparative Ct (threshold cycle) method according to the manufacturer’s instructions (User Bulletin No. 2; ABI PRISM Sequence Detection System). The parameter Ct is defined as the cycle number at which intensity of fluorescence (which is proportional to the quantity of DNA present in the tube during the exponential phase of the PCR) passes a fixed threshold value. The relative amount of target in two preparations containing the same quantity of cellular RNA is 2^{ΔCt} condition A − Ct condition B, where conditions A and B were normal human serum and decenplemented serum, respectively. Experiments were performed three times. Dilutions of the reverse transcription product were loaded in duplicate for each oligonucleotide couple in each experiment.

Microarray data analysis. Images were revealed with a STORM phosphorimager (Molecular Dynamics) and interpreted with the software XdotsReader (COSE France), which quantified the intensity of the signal associated with each spot. The background level of reactivity was measured as the mean value of the amplicons corresponding to mRNA.
to ORFs from *N. meningitidis* strain MC58 shown by BLAST comparison to have less than 10% nucleotide identity to sequences from the Z2491 genome. This background was subtracted (negative values were corrected to zero), then the level of expression of a gene relative to the mean was calculated by normalization with respect to the mean reactivities of the expressed genes. To avoid artificially large ratios, those amplicons which had given zero or negative values after subtraction of the background were accorded a value of 1/15 (<0.0667). These background-subtracted, normalized values were used to calculate ratios of expression of each gene in bacteria that were or were not exposed to the signalling molecule.

Analysis was based on the results from three independent experiments with and without exposure to AI-2. Manual analysis of the normalized data demonstrated good reproducibility: 90% of the groups of values for any one gene in one condition (means of duplicate spots on three DNA arrays) had a standard deviation less than 20% of the mean, while 95% of the genes had a standard deviation less than 25% of the mean. Calculated ratios from three independent experiments were analysed using Significance Analysis of Microarrays (SAM) Version 1.15 (http://www-stat.stanford.edu/~tibs/SAM/) (Tusher et al., 2001), a program which compares gene-specific tests and assigns a score based on the change in gene expression and the standard deviation of repeated measurements. For the statistical analysis of high abundance transcripts the relative expression levels for subsets of genes were compared to that for the rest of the chromosome using the Mann–Whitney test.


**RESULTS**

**Identification and characterization of the luxS homologue of serogroup A *N. meningitidis* strain Z2491**

Analysis of the genome sequence of strain Z2491 revealed a hypothetical protein (NMA0463) with 80% identity and 89% similarity to the LuxS protein from *V. harveyi*. This protein is also conserved in the three other strains of *Neisseria* for which the genome sequence is available, two of *N. meningitidis* (MC58 and FAM18) and one of *N. gonorrhoeae* (FA1090), and appears organized as part of an operon (Fig. 1). Indeed, a recent DNA array analysis of the full genome content of *Neisseria* species showed that all the genes shown with the exception of NMA0465 were present in all of eight *N. meningitidis*, three *N. gonorrhoeae* and two *Neisseria lactamica* strains tested (Perrin et al., 2002), suggesting that the enzyme is common to *Neisseria* species.

AI-2 activity was detected by its ability to induce luminescence in *V. harveyi* (Bassler et al., 1993, 1994). A *V. harveyi* mutant denoted BB170 (sensor 1–sensory 2+) that increases luminescence specifically in response to AI-2 has been constructed as a reporter strain for detecting AI-2 (Bassler et al., 1993, 1994). Cell-free supernatants of exponential-phase cultures of *N. meningitidis* Z2491 (serogroup A) were found to induce luminescence of *V. harveyi* BB170 to 35% of the level induced by supernatants of wild-type *V. harveyi* (BB152). Similar levels of AI-2 production were observed for meningococci grown in RPMI supplemented either with serum or with BSA. Similar data

**Fig. 1.** Genetic organization of the gene luxS in the publicly available neisserial genome sequences. The luxS gene appears to be the last ORF in a potential operon consisting of four genes in *N. meningitidis* strains Z2491 and MC58, and of three in *N. meningitidis* strain FAM18 and in *N. gonorrhoeae* FA1090. In each case it is terminated by a hairpin loop structure composed of an inverted repeat of neisserial uptake sequences, as is common in the pathogenic *Neisseria* species.
were obtained with strain 8013-2C43, a serogroup C meningococcus.

To confirm that the luxS gene was necessary for production of AI-2, we engineered mutants in two strains of *N. meningitidis*, Z2491 and 8013-2C43. Cell-free supernatants from exponential-phase luxS mutants of *N. meningitidis* stimulated luminescence to levels 0-4% of that induced by supernatants of wild-type *V. harveyi* BB152 (in comparison to 35% for wild-type *N. meningitidis*), this being not significantly higher than the luminescence resulting from medium alone. Taken together, these data show that these strains produce an AI-2 activity that requires the luxS gene, in agreement with a previous study with the serogroup B strain MC58 (Winzer et al., 2002b).

In other bacteria, including *H. pylori* (Forsyth & Cover, 2000; Joyce et al., 2001) and serogroup B *N. meningitidis* (Winzer et al., 2002b), the autoinducer AI-2 molecule has been shown to be produced predominantly during mid to late-exponential-phase growth. To investigate the kinetics of production of AI-2 in the strain used in our experiments, cell-free supernatants were obtained from cultures at early, mid and late exponential phase. The results demonstrate that per bacterium AI-2 production is maximal at mid-exponential phase and is greatly diminished both before and after this point (Fig. 2).

**AI-2 does not affect growth in medium or in contact with epithelial cells**

Consistent with a growth-phase-dependent production of AI-2, luxS mutants of both *E. coli* (Sperandio et al., 2001) and *Str. pyogenes* (Lyon et al., 2001) have been shown to have different doubling times relative to the wild-type strain. In contrast, for *N. meningitidis* serogroup A and C, the growth of the wild-type and mutant strains in both rich medium and RPMI supplemented with serum are indistinguishable (data not shown), as has been observed for serogroup B.

An essential step in the interaction of meningococci with their host is their ability to interact with human cells. Infection of epithelial cells by *N. meningitidis* involves an initial clumping stage, in which bacteria form microcolonies on the surface of the epithelial cell monolayer, followed by a gradual dispersion of the bacteria to form a tightly adhering monolayer (Pujol et al., 1997, 1999). The infection of epithelial cells by wild-type bacteria and the luxS mutant were compared to determine whether the luxS quorum sensing system was important in the progression of bacterial adhesion. For both serogroup A and C, the luxS mutant and wild-type bacteria showed identical behaviour during the infection process: bacterial strains were indistinguishable in the percentage of inoculum that adhered to epithelial cells, the formation of initial clumps and in the rate of spreading to form a monolayer (data not shown).

**Gene array analysis of AI-2-induced transcription**

In *E. coli*, genomic microarrays have been used successfully to monitor changes in levels of gene expression in response to quorum sensing and have shown that AI-2 has a global regulatory effect on diverse processes, including chemotaxis, flagellar expression and cell division (DeLisa et al., 2001; Sperandio et al., 2001). To determine the effect of AI-2 on gene expression in *N. meningitidis*, exponential-phase cultures of the luxS mutant were collected and resuspended in either cell-free supernatants from wild-type (containing AI-2) or the luxS mutant (without AI-2). Following incubation for 30 min, gene expression was analysed by hybridization of extracted RNA (the transcriptome) to *N. meningitidis* DNA arrays.

To determine significant AI-2-induced transcriptional changes over the whole genome the ratio of gene expression in the presence and absence of AI-2 was analysed. The results clearly reflected very little gene regulation in response to CM containing AI-2 with 96% having a ratio of less than twofold, 99% of the genes having a ratio of less than threefold and no genes differing by more than sixfold in bacteria exposed to CM with and without AI-2 (Fig. 3; Table 1; see supplementary data at http://mic.sgmjournals.org for a complete list of changes in expression of genes). To test the significance of these changes, the data were subjected to analysis using the computer program SAM (Tusher et al., 2001), commonly used for the analysis of transcriptomic data. This analysis showed only one gene, NMA0452 (Parkhill et al., 2000), a possible iron-siderophore receptor, to be significantly overexpressed. (Employing the parameters where this gene was indicated, SAM also predicted the presence of one false positive;
there were no genes significantly down-regulated.) This result is in dramatic contrast to E. coli where 1–10% of the genome changed by more than fivefold, in the two published reports, and expression ratios up to 50 were observed (DeLisa et al., 2001; Sperandio et al., 2001). In these studies, the work of Sperandio and colleagues compared wild-type to luxS bacteria, while that of DeLisa’s group compared, as we did, the response of the bacteria to exogenous AI-2 in CM. It is clear that, under the same conditions, the response of N. meningitidis to AI-2 differs greatly from that of E. coli.

This unexpected result led us to examine the expression data more closely to validate these results. To show that the arrays were capable of quantifying the range of transcript abundance present in the samples, we looked at the levels of expression of each gene, concentrating on those which are known to have high levels of expression under normal conditions, i.e. those for ribosomal and major outer-membrane proteins (OMPs) (Wei et al., 2001). As expected, these genes are highly expressed by N. meningitidis grown under normal conditions (Table 2; data from the experiments described above). Comparing the levels of expression of these groups of genes (56 ribosomal protein genes, 16 OMP genes) with that of the other 2005 genes, the OMP genes were expressed at a mean of 11 times the level of the rest of the chromosome (P<0.0001), while the ribosomal protein genes were 5–6 times overexpressed (P<0.0001) (Table 2). This observation shows that under the experimental conditions used in this study, the DNA array is indeed capable of detecting differences in levels of transcripts in the RNA samples taken from autoinducer-exposed and non-exposed meningococci.

We further tested the ability of the DNA arrays to detect changes in transcription levels of meningococcal genes by using them to analyse RNA expression patterns under conditions where the meningococci did indeed respond to the growth conditions, results being confirmed by real-time PCR performed on reverse transcriptase-generated cDNA. N. meningitidis strain Z5463, acclimatized to low-iron conditions was grown either in serum containing a functional complement system or in heat-inactivated serum. Changes in RNA expression levels were analysed using the DNA arrays. The results were analysed as above using the program SAM. Of those genes whose expression changed in response to attack by the complement system, we chose three highly expressed genes for further analysis (Table 3). To confirm these DNA array results, primers for each gene, and for a control unaffected gene, were designed to produce cDNA from the RNA samples and the corresponding transcripts were quantified by real-time PCR using the Sybr-Green system. The results were concordant (Table 4), demonstrating that the DNA arrays are capable of detecting changes in expression of the genes, and that the negative result obtained for the AI-2 experiments does indeed represent an absence of response to the autoinducer.

**Fig. 3.** Transcriptional changes in response to CM containing Al-2. The log of the ratio of expression of each gene in the presence of Al-2 relative to the absence of Al-2 was determined. As expected, no change between the two conditions is most frequently observed. No changes above a 0·8 or below −0·8 (a change of sixfold in either direction) were observed.

**DISCUSSION**

The LuxS-dependent quorum sensing system could be an attractive target for antibiotics since the structure of the biosynthetic enzyme for the signalling molecule is known and a role in virulence has been demonstrated, notably in E. coli (Sperandio et al., 1999), V. cholerae (Miller et al., 2002) and Str. pyogenes (Lyon et al., 2001). However, many details of the quorum sensing system remain to be elucidated, including the physiological response induced by AI-2 in the large number of bacteria containing the luxS gene, and therefore likely to produce AI-2. Additionally, an identical, or very similar, signalling molecule appears to be produced by a wide variety of bacterial species. The observation of cross-species induction of luminescence in V. harveyi demonstrates that the signalling molecule from one species of bacterium is able to cause a physiological response in other species (Bassler et al., 1997; Schauder & Bassler, 2001). This will certainly have repercussions for bacterial ecology that have only just begun to be investigated.

Observation of a putative LuxS homologue in the serogroup B N. meningitidis genome suggested that quorum sensing could be important for pathogenesis and indeed a recent report gave support to this hypothesis by demonstrating that a luxS mutant was not as fit as the wild-type strain during bloodstream dissemination in infant rat (Winzer et al., 2002b). To characterize a possible quorum sensing role for AI-2 in N. meningitidis, we used a serogroup C strain (8013-2C43) because of its well characterized interaction with epithelial cells, and a serogroup A strain (Z2491) because the availability of its genomic sequence
permitted us to produce a DNA array (Perrin et al., 2002). Our results have now confirmed the presence of a LuxS-dependent AI-2 activity, with growth-phase-dependent production of autoinducer in both of these strains of N. meningitidis.

As a first step in characterizing the putative quorum sensing system, and considering that the meningococcus is a commensal of the nasopharynx, we concentrated on phenotypes related to the normal lifestyle of N. meningitidis, such as infection of epithelial cells. Despite extensive investigation, no phenotypic differences were observed between the luxS mutant and the wild-type bacteria during the infection of epithelial cells or during growth in liquid culture. Since AI-2 has been shown to be involved in regulating the transcription of a number of genes (Bassler et al., 1993, 1994; Day & Maurelli, 2001; DeLisa et al., 2001; Lyon et al., 2001; Sperandio et al., 1999, 2001), we then turned to DNA microarrays to identify genes whose transcription was regulated in response to CM containing AI-2 in N. meningitidis. Surprisingly, we found little or no significant transcriptional changes. The SAM program (Tusher et al., 2001), showed only one gene whose transcription levels were significantly regulated. This gene, NMA0452 (Parkhill et al., 2000), shows homology to proteins involved in iron acquisition; such systems have been reported to be affected by quorum sensing in some other species (Fong et al., 2001). However, the parameters used to demonstrate this positively regulated gene were also predicted by the program to result in one false positive, and hence the relevance of this change is uncertain. Although it is always possible that under different conditions a response to AI-2 might be observed, these data suggest that AI-2 is not functioning as a quorum sensing molecule in the meningococcus. This result is consistent with the absence of a LuxP homologue.

### Table 1. Genes whose expression changed by more than a factor of two in response to CM containing AI-2

Although these genes demonstrated an apparent change in transcript level, only one such change (that of NMA0452) was found to be statistically significant by using SAM analysis (see Results). In addition, the program suggested a false positive discovery rate of one gene.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Expression ratio</th>
<th>Gene, possible function or homology*</th>
</tr>
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<tbody>
<tr>
<td>NMA1355</td>
<td>5.86</td>
<td>Possible periplasmic protein</td>
</tr>
<tr>
<td>NMA1757</td>
<td>5.75</td>
<td>holC, DNA polymerase III chi subunit</td>
</tr>
<tr>
<td>NMA1654</td>
<td>5.55</td>
<td>Unknown</td>
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<tr>
<td>NMA2052</td>
<td>4.40</td>
<td>acaA, aconitate hydratase</td>
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<tr>
<td>NMA0869</td>
<td>3.86</td>
<td>tmk, probable thymidylate kinase</td>
</tr>
<tr>
<td>NMA0429</td>
<td>0.26</td>
<td>vsr, very short repeat patch repair protein</td>
</tr>
<tr>
<td>NMA0024</td>
<td>0.27</td>
<td>Unknown</td>
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<tr>
<td>NMA1129a</td>
<td>0.27</td>
<td>Possible pseudogene</td>
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<td>Probable integral membrane protein</td>
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<tr>
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<td>NMA2047</td>
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<td>Hypothetical protein</td>
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</table>

*Annotation according to Parkhill et al. (2000).
Table 2. High expression levels of OMP and ribosomal protein genes

Results are from an analysis of the DNA array results of non-AI-2-treated meningococci. Similar results were obtained, as expected, from analysis of the AI-2-treated bacteria. Levels of expression were calculated by dividing the intensity of signal obtained after hybridization with labelled RNA by that obtained after hybridization with labelled chromosomal DNA.

<table>
<thead>
<tr>
<th>Gene*</th>
<th>Relative expression†</th>
<th>Rank‡</th>
<th>Gene*</th>
<th>Relative expression†</th>
<th>Rank‡</th>
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<td>rmp</td>
<td>36·6</td>
<td>3</td>
<td>pilS7</td>
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<td>67</td>
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<tr>
<td>porB</td>
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<td>4</td>
<td>opaD</td>
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<td>pilS5</td>
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<td></td>
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<tr>
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<td>108</td>
<td>rpmD</td>
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</table>

*In the meningococcal genome there are multiple pilin (pil) and opacity protein (opa) genes (8 and 5 respectively for strain Z2491). Only one of the pil genes is downstream of a promoter and thus expressed, while all opa genes are expressed, regulation being achieved by phase shifting. Hence some of the apparent high relative expression levels of the opa genes may be explained by the simultaneous transcription of several genes. There is some homology between the genes porA and porB, but the other genes are present in single copy in the meningococcal genome.

†Results shown are the level of expression of a gene relative to the mean over each gene in the chromosome.

‡The rank level of expression is given among the 2077 genes represented on the membrane. e.g. rmp (the gene for the class 4 OMP) is the 3rd most highly expressed of the 2077 genes.

AI-2 responsive system has been described which regulates flagellar and chemotactic genes in *E. coli*, where the autoinducer is recognized by a two-component sensory transduction system, QseB/C (Sperandio et al., 2002). The four publicly available genomes of the pathogenic *N. meningitidis* species contain predicted proteins with weak similarity to the sensor/kinase component (mean 24 % amino acid identity, 38 % similarity) and to the regulator component (mean 33 % amino acid identity, 49 % similarity). However, for the sensor/kinase component, the homology was restricted to the C-terminal, cytoplasmic signal-transducing domains, while the N-terminal sensor domain showed very
little similarity (data not shown). Hence, we found no evidence for the presence of an AI-1 receptor in *Neisseria meningitidis*.

Recently reported work suggests that AI-2 plays a role in bacteraemia caused by *N. meningitidis* (Winzer *et al.*, 2002b). Although a difference was observed in the levels of wild-type and *luxS* mutant bacteria during a competitive infection in an infant rat model, the explanation for this observation is not known. AI-2 functioning as a quorum sensing molecule, however, is unlikely to explain the result because in the competitive infection, the wild-type and *luxS* mutant bacteria were injected as a 1:1 mixture and would have experienced the same concentration of any signalling molecule throughout the course of the infection. This hypothesis would be in agreement with our results, which show no reaction to externally provided AI-2. It is possible that the mutant cells suffer some metabolic and hence physiological dysfunction which leads to a reduced ability to resist immune attack. It may be that this physiological change resulting from the absence of LuxS is not detectable under the conditions of normal growth and interaction with epithelial cells, but becomes important in the later stages of the infection.

Given that *N. meningitidis* clearly produces AI-2, it is interesting to consider what role the molecule serves. One explanation is that AI-2 is simply a metabolite that is

<table>
<thead>
<tr>
<th>Gene†</th>
<th>Overexpression factor</th>
<th>Gene†</th>
<th>Overexpression factor</th>
<th>Gene†</th>
<th>Overexpression factor</th>
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<td>NMA0445</td>
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<td></td>
<td></td>
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</tr>
</tbody>
</table>

†Genes shown by SAM analysis to be significantly overexpressed in response to human serum in strain Z2491. Genes marked with an asterisk were verified by real-time PCR.

Table 3. Genes more than fivefold overexpressed in the presence of complement, as measured by DNA array

<table>
<thead>
<tr>
<th>Gene†</th>
<th>Overexpression factor (DNA array) ± SD*</th>
<th>Mean relative expression (real-time PCR assay) ± SD*</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMA1161</td>
<td>23.4 ± 4.2</td>
<td>16.7 ± 1.3</td>
</tr>
<tr>
<td>NMA1686</td>
<td>44.6 ± 26.1</td>
<td>23.2 ± 6.2</td>
</tr>
<tr>
<td>NMA1852</td>
<td>6.9 ± 1.3</td>
<td>6.3 ± 2.2</td>
</tr>
<tr>
<td>NMA1203†</td>
<td>0.8 ± 0.1</td>
<td>1.5 ± 0.4</td>
</tr>
</tbody>
</table>

*SD of the three independent experiments.
†NMA1203 did not show a significant difference in expression between the two conditions and is included as a control.

Table 4. Validation of the DNA array results: confirmation of expression ratios of genes overexpressed in the presence of complement by real-time PCR of cDNA

additionally utilized by some bacteria, such as V. harveyi, as a cell signalling molecule. Again, the ability of the wild-type and luxS mutant strain to grow in medium and infect epithelial cells equally well, suggests that the production of AI-2 does not influence cell viability. In addition we observed no changes in transcription, which one would have expected to accompany a metabolic change if AI-2 were an obligate by-product of an essential metabolic process, as has been shown for some other bacteria (Winzer et al., 2002a). Although additional experiments examining the metabolic pathway resulting in AI-2 production will be necessary, our results do not support the idea that the production of AI-2 serves a metabolic role in meningococcus.

If N. meningitidis does not regulate gene expression in response to the signalling molecule or serve as an important metabolite, what is the reason for AI-2 production? Considering the large number of bacterial species which colonize the nasopharynx, the natural habitat for N. meningitidis, AI-2 produced by N. meningitidis would be detected by other bacterial species. This type of interspecies communication has already been demonstrated in oral bacterial ecosystem (reviewed by Kolenbrander et al., 2001). In the case of AI-2, production of the signalling molecule by different bacterial species is believed to play a role in interspecies interactions in the oral bacterial ecosystem (reviewed by Kolenbrander et al., 2002). An attractive hypothesis is that N. meningitidis can benefit by production of AI-2, for example if it caused other bacteria to slow their growth rates prematurely or resulted in the production of secreted factors that aided its colonization of the nasopharynx.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


