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Global Gene Expression and the Role of Sigma Factors in *Neisseria gonorrhoeae* in Interactions with Epithelial Cells

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Like many bacterial pathogens, *Neisseria gonorrhoeae* must adapt to environmental changes in order to successfully colonize and proliferate in a new host. Modulation of gene expression in response to environmental signals is an efficient mechanism used by bacteria to achieve this goal. Using DNA microarrays and a tissue culture model for gonococcal infection, we examined global changes in gene expression in *N. gonorrhoeae* in response to adherence to host cells. Among those genes induced upon adherence to human epithelial cells in culture was *rpoH*, which encodes a homolog of the heat shock sigma factor, σ^{32} (RpoH), as well as genes of the RpoH regulon, *groEL* and *groES*. Attempts to construct an *rpoH* null mutant in *N. gonorrhoeae* were unsuccessful, suggesting that RpoH is essential for viability of *N. gonorrhoeae*. The extracytoplasmic sigma factor, RpoE (σ^E), while known to regulate *rpoH* in other bacteria, was found not to be necessary for the up-regulation of *rpoH* in gonococci upon adherence to host cells. To examine the role of RpoH in host cell interactions, an *N. gonorrhoeae* strain conditionally expressing *rpoH* was constructed. The results of our experiments showed that while induction of *rpoH* expression is not necessary for adherence of gonococci to epithelial cells, it is important for the subsequent invasion step, as gonococci depleted for *rpoH* invade cells two- to threefold less efficiently than a wild-type strain. Taken together, these results indicate that σ^{32}, but not σ^E, is important for the response of gonococci in the initial steps of an infection.

Neisseria gonorrhoeae (gonococcus), an obligate human pathogen, is the causative agent of the sexually transmitted disease (STD) gonorrhea. Gonococcal disease is prevalent worldwide, with ~500,000 reported cases each year in the United States alone. It is estimated that at least as many additional cases go unreported. While aggressive safe sex practice campaigns significantly reduced the incidence of several STDs in the 1980s and 1990s, the last 5 years have shown a gradual increase in gonorrhea as well as another bacterial STD, syphilis (Centers for Disease Control and Prevention [http://www.cdc.gov/std/stats/toc2002.htm]). There are several challenges in the treatment of gonococcal disease, including continuing acquisition of antibiotic resistance (2, 19, 74), a high incidence of asymptomatic infection (especially in women), and the observation that gonococcal infection does not elicit protective immunity (68). Patients can be reinfected following treatment and can even be infected by multiple strains at a given time. Thus, understanding the biology of this organism and how it senses and responds to its environment will be key in the development of alternative treatments and preventative strategies for gonococcal infection.

Gonorrhea is typically a disease of the urogenital tract, although the bacterium can also infect tissues of the pharynx, rectum, and the conjunctiva (which can lead to blindness in newborns, who are infected during birth). The initial step of a gonococcal infection is colonization of mucosal tissues. Following adherence to mucosal epithelial cells, the bacteria then enter the cells and traverse across them to exit to the subepithelial space, where they elicit an acute inflammatory response, resulting in the symptoms characteristic of gonorrhea. In some cases, the organism will ascend from the site of infection into the fallopian tubes, resulting in gonococcal salpingitis, a leading cause of infertility in women. It will also occasionally spread to other tissues in both men and women, resulting in a full-blown bacteremia, termed disseminated gonococcal infection, a major sequela of which is arthritis.

The colonization of mucosal tissues by *N. gonorrhoeae* is a critical first step in a gonococcal infection. Adherence to cells of the mucosal epithelia is essential to prevent the bacterium from being washed away by urine (in the male urethra) or vaginal fluid (in the female genital tract). Colonization of the host epithelia by gonococci is a complex process involving multiple components of both the bacterial and host cell. The incoming bacterium first binds to epithelial cells via interactions between specific receptors on the bacterial and host cell surfaces. The primary adhesins on the gonococcal cell surface are the type IV pili (77, 78), although there are additional surface structures of the bacterium that can participate in adherence (18, 22, 33, 37, 46, 47, 67, 82, 84). Binding of gonococcal pili to host cells results in the induction of signal transduction pathways in the eukaryotic cell that affect multiple cellular processes (4, 34, 43, 57, 58, 62). Adherence of piliated gonococci to epithelial cells also results in a variety of rearrangements in the components of the cellular cytoskeleton (reviewed in reference 50) as well as significant changes in host cell gene expression (61). However, our understanding of gonococcal infection with respect to the specific response of *N. gonorrhoeae* at the level of gene expression is still incomplete.

Our hypothesis is that the initial contact between gonococci and host epithelial cells signals the bacterium to modulate the

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expression of genes that will allow it to colonize and proliferate in the new host. In this work, we sought to identify genes that are triggered by the adherence of gonococci to host cells and to identify regulatory phenomena that are involved in this response. Using a tissue culture model of infection and gonococcal DNA microarrays, we examined changes in patterns of gene expression in the gonococcus in response to contact with human epithelial cells in culture. Here we report that *rpoH*, which encodes RpoH, a homolog of the heat shock sigma factor, σ^{32}, as well as genes expected to be controlled by RpoH, are induced after host cell contact. Further studies show that *rpoH* is essential to *N. gonorrhoeae* and is important for the invasion of, but not adherence to, human epithelial cells. These data therefore suggest a role for the heat shock regulon in gonococcal pathogenesis.

MATERIALS AND METHODS

Growth and construction of bacterial strains. *Escherichia coli* strain DH5α was used for all recombinant DNA manipulations (65) and was grown in Luria broth supplemented as necessary with ampicillin (Ap) at 100 mg/liter, chloramphenicol (Cm) at 20 mg/liter, kanamycin (Kn) at 50 mg/liter, or erythromycin (Em) at 300 mg/liter.

N. gonorrhoeae strain MS11 [P^+ Tr (69)] was grown in a humidified 5% CO_2 atmosphere in GC medium (Difco Laboratories, Sparks, MD) with supplements (36). When necessary, antibiotics (Em and Cm) were added at 3 mg/liter and 6 mg/liter, respectively. *N. gonorrhoeae* transformation and genomic DNA isolation were performed as described elsewhere (69, 71). Gonococcal strains used in these studies were predominantly Opa^- and P^+ as determined by colony microscopy.

DNA manipulations. Cloning vectors used were pHSS6 (72), pDONR221 (Invitrogen, Carlsbad, CA), pET24a (Novagen, Madison, WI), and pKH35 (27). Restriction enzymes, T4 DNA ligase (New England Biolabs, Beverly, MA), and Gateway PCR cloning kits (Invitrogen) were used according to the manufacturers' recommendations. PCR was done with *Taq* DNA polymerase (purified as described elsewhere [17]) in a GeneAmp 9700 thermocycler (Applied Biosystems, Foster City, CA). When necessary, amplification products were purified using the QIAquick PCR purification kit (QIAGEN, Valencia, CA). Oligonucleotide primers were synthesized at the Michigan State University Macromolecular Structure, Sequencing and Synthesis Facility (MSU GTSF; sequences available on request). DNA sequence determination was done by the MSU GTSF and analyzed using the Oxford Molecular Group DNA analysis program Omiga (Accelrys, San Diego, CA). Transposon mutagenesis was performed by in vitro transposition using EZ::TN transposase (Epicentre Technologies, Madison, WI) and the modified transposon TnErmUP as described previously (16). The positions of transposon insertions were determined by PCR using primers homologous to the ends of the transposon (SqFP and SqRP; Epicentre) and to the ends of the gene in question and corroborated by restriction analysis.

Cell culture. Human epithelial cell line A431 (ATCC CRL 1555) was grown at 37°C in a humidified 5% CO_2 atmosphere in Dulbecco modified Eagle medium (DMEM; Gibco/Invitrogen) supplemented with 5% fetal calf serum (FCS; Gibco). Adhesion and invasion assays were performed as described previously (3, 82). Briefly, gonococci grown on GC agar (16 to 20 h cultures) were swabbed into GC broth, and the concentration was determined spectrophotometrically. Bacteria were diluted in DMEM–5% FCS to appropriate concentrations, and the bacterial suspension was added to A431 cells grown to 50 to 90% confluency in 24-well or 100-mm tissue culture plates as indicated below. Serial dilutions of each bacterial inoculum were diluted and plated on GC agar plates to determine the actual number of CFU added. Infected cells were incubated at 37°C with 5% CO_2 for 3 h (for adherence assays) or 7 h (for invasion assays). Following incubation, the samples were divided into two sets (for adherence assays) or three sets (for invasion assays). For the first set (total CFU), bacteria in the supernatant were transferred to a sterile tube. Cells (with adherent bacteria) were lifted with saponin (1% in GC broth), this suspension was added to the bacteria from the supernatant, and serial dilutions were plated onto GC agar. This represents the total number of CFU in the well at the end of the experiment, a necessary control since gonococci multiply in the cell culture medium throughout the experiment and adherence is measured as a function of total number of bacteria at the end of the experiment. For the second set (cell-associated CFU), infected cells were washed with phosphate-buffered saline (PBS) to remove nonadherent bacteria and cells (with adherent bacteria) lifted with 1% saponin in GC broth. Serial dilutions were plated to determine cell-associated CFU. The ratio of cell-associated CFU to total CFU at the end of the experiment was defined as the adhesion frequency. For invasion assays, a third set of wells were washed to remove nonadherent bacteria (as above) and fresh DMEM–5% FCS containing 50 mg/liter gentamicin (Gm^{50}) was added to each well to kill extracellular bacteria. Following incubation at 37°C with 5% CO_2 for 1 h, Gm-containing medium was removed, cells were washed with PBS, and cells were lifted with 1% saponin in GC broth. Serial dilutions were plated to determine intracellular CFU. The ratio of Gm^r CFU to cell-associated CFU at the end of the experiment was defined as the invasion frequency.

RNA isolation. RNA was isolated from adherent bacteria or bacteria grown in the absence of cells by using TRIzol reagent (Invitrogen). RNA was quantified spectrophotometrically, and quality was assessed by agarose gel electrophoresis.

Construction of gonococcal DNA arrays. DNA microarrays were made by amplification of 2,043 open reading frames (ORFs) of the *N. gonorrhoeae* genome, 1,985 from strain FA1090 (8, 13), and 58 from the gonococcal genetic island of *N. gonorrhoeae* strain MS11 (14, 27). Sizes of the amplicons spotted ranged from 143 bp to 3,485 bp in length and corresponded to the predicted ORF of each gene. An additional 362 amplicons corresponding to internal ORF sequences were generated for genes with insufficient DNA observed upon agarose gel electrophoresis of the initial PCR. PCR amplicons were spotted in duplicate onto SuperAmine glass slides (TeleChem International Inc., Sunnyvale, CA) at the MSU GTSF. The presence of DNA on the arrays was validated by hybridization with a Cy3-labeled random nonamer oligonucleotide (QIAGEN). A scan of the slide at 532 nm showed spots at the appropriate positions where DNA had been spotted and blank spots at the buffer control spots (data not shown). DNA hybridization of the arrays with MS11 genomic DNA showed valid hybridization signals (above background) for 98% of the genes spotted on the slides and indicated that these arrays would be suitable for the analysis of gene expression in *N. gonorrhoeae* strain MS11. (A manuscript detailing the construction and use of the arrays has been submitted for publication.)

Microarray hybridization. Ten micrograms of total bacterial RNA was labeled using the CyScribe first-strand labeling kit (Amersham Biosciences, Piscataway, NJ), which employs random primed reverse transcription in the presence of Cy3- or Cy5-dCTP. Cy3- and Cy5-labeled cDNAs were purified on QIAquick Cleanup columns (QIAGEN) and concentrated using Microcon YM-30 microcentrifugation units (Millipore, Billerica, MA).

DNA array slides were prehybridized at 42°C in prehybridization buffer (5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% sodium dodecyl sulfate [SDS], 1% bovine serum albumin). For hybridization, Cy3- and Cy5-labeled probes were mixed (10 μl each) with 1 μl of 1-mg/ml sheared, sonicated herring sperm DNA and denatured by boiling for 5 min, followed by chilling on ice. Twenty microliters of 4× hybridization buffer (Amersham) and 40 μl of formamide were added to the denatured DNA, and the entire hybridization mix was pipetted onto the prehybridized array slide. Following hybridization overnight at 42°C, the coverslips were removed by gently immersing in the first wash (1× SSC, 0.2% SDS). Washes were once in 1× SSC, 0.2% SDS at 42°C, twice in 0.1× SSC, 0.2% SDS at room temperature, and once in 0.1× SSC. Slides were then dried and scanned using a GenePix 4000B scanner (Axon Instruments, Union City, CA), and images were processed and analyzed using GenePix version 4.1 software.

DNA array data analysis. Data from four independent experiments (biological replicates), including one in which the dyes were swapped, were normalized to eliminate labeling artifacts (intensity-based normalization) and expression ratios were determined. In these four hybridization experiments, 75 to 85% of the spots on the slide had at least 40% of the pixels >1 standard deviation above background in at least one channel (535- or 632-nm wavelength). Spots not above this cutoff were not included in the normalization or outlier analyses. Outliers were identified by iterative outlier analysis, in which three successive rounds of analysis are done to determine genes with ratios ($\log_2$ transformed) greater than 2.5 standard deviations from the mean (9). Confidence limits of the data were calculated, including a standard error factor (distance from gene's average value to the nearest 2.5 standard deviation cutoff) and the number of standard deviations separating the spot from the 2.5 standard deviation cutoff. These data are not included in the tables but were taken into consideration when determining genes for which data from multiple spots or experiments were not consistent.

RT-PCR. Total bacterial RNA was prepared as described above. cDNA was synthesized from 100 ng RNA with Superscript II RNase H^- reverse transcriptase (RT; Invitrogen) using random nonamers (Sigma-Aldrich, St. Louis, MO). Gene-specific primers for *rpoH*, *groES*, and 16S rRNA (internal control) were used in the subsequent PCR, which was carried out for 30 cycles. Negative controls for each experiment included a no-template control and a no-RT con-

trol where RNA was added as a template for the final PCR (to rule out genomic DNA contamination of RNA preparations). Products were analyzed by agarose gel electrophoresis and stained with ethidium bromide (EtBr) for photographing and/or quantification using KODAK 1D image analysis software (Eastman Kodak, Rochester, NY).

RESULTS

Analysis of global gene expression in gonococci adherent to cells in culture. Several cell lines have been used to characterize interactions between gonococci and host cells (3, 11, 30, 31, 35, 40, 48, 49, 55, 73, 82). Each cell line has slightly different features with respect to specifics of interactions with gonococci and handling in the laboratory. For the experiments described in this work, A431 cells (20), an epithelial cell line derived from the endocervix (56), were used. Gonococci have been shown to adhere to and invade A431 cells at a high frequency (3, 30, 43, 48, 76), and there have been several reports investigating the response of these cells to gonococcal adherence at the molecular level (4, 5, 7, 43).

Total RNA was isolated from A431 cells alone, bacteria alone (MS11), and A431 cells infected with MS11 for 3 h. A 3-h infection time was chosen since this has been reported as the minimum time where most gonococci that will adhere to cells have done so, yet few if any have invaded (73). cDNA was synthesized from each RNA incorporating digoxigenin-11-dUTP (Roche Applied Science, Indianapolis, IN) and used to probe a Southern blot of ClaI-digested MS11 genomic DNA (3). The results of this experiment (data not shown) showed that cDNA from bacteria alone and cells infected with bacteria gave a strong signal following chemiluminescent detection, with intense bands at positions corresponding to the bacterial rRNAs and a fainter banding pattern (upon extended exposure) that was presumably from mRNA. cDNA from uninfected A431 cells gave no signal at all, even after prolonged exposure. This indicated that any remaining eukaryotic RNA in the infected cell samples (minimal, since the cells are lysed before bacteria are harvested) would not create a background problem in DNA array hybridizations.

We next analyzed global gene expression in gonococci adherent to A431 cells. *N. gonorrhoeae* strain MS11 was used to infect A431 cells at a multiplicity of infection (MOI) of 100. An MOI of 100 was chosen to ensure that sufficient quantities of RNA would be isolated for hybridization experiments. At 3 h postinfection (p.i.), the cells were washed with PBS to remove nonadherent bacteria. Cells (with adherent bacteria) were lifted with 1% saponin in GC broth, and samples were diluted for plating to determine cell-associated CFU. Duplicate plates containing media without cells were "infected" as the bacteria-alone sample, and bacteria were harvested without washing. Adhesion frequencies were determined by comparing the number of adherent CFU to the total number of CFU (CFU in the supernatant plus adherent CFU) at the end of the experiment. The total number of CFU in wells containing A431 cells was comparable to the number of CFU in the bacteria-alone control, indicating that the growth of gonococci was not appreciably altered in the presence of epithelial cells. Adhesion frequencies varied from 40 to 66%, comparable to previous reports of gonococcal adherence under similar conditions at an MOI of 10 (3, 30, 48). Bacteria from both samples (adherent bacteria and bacteria alone) were concentrated by centrifugation, and RNA was isolated immediately using TRIzol (Invitrogen). Labeled cDNA probes were generated from isolated RNA and hybridized to DNA arrays as described in Materials and Methods.

Global gene expression in adherent gonococci was compared with that of gonococci grown in cell culture medium in the absence of A431 cells at 3 h p.i. Four independent experiments were performed, and outliers were identified by iterative outlier analysis, in which three successive rounds of analysis are done to determine genes with ratios (log_2 transformed) greater than 2.5 standard deviations from the mean (9). Averages of ratios (and standard deviations) of genes identified as outliers in at least two experiments as differentially regulated by host cell contact are shown in Tables 1 (up-regulated) and 2 (down-regulated).

Of the 94 genes identified as differentially regulated, several genes (35/94) encode hypothetical proteins. This was not surprising, as nearly half of the genes in the annotated genome of *N. gonorrhoeae* encode putative proteins with no similarities to proteins of known function (8). The remaining 59 genes identified as differentially regulated encode proteins with a broad spectrum of functions in *N. gonorrhoeae*, including amino acid metabolism, protein export, transcription, translation, protein modification, glycolysis, oxidation-reduction reactions, cell division, and replication.

A control microarray experiment was done to determine whether saponin treatment (used to lift cells with adherent bacteria) affected gonococcal gene expression. The results of this experiment showed no genes were significantly down-regulated and six genes (NG0387, NG0652, NG1055, NG1767, NG1779, and NG2065) were consistently up-regulated in bacteria treated with saponin for 10 min (data not shown). However, none of the genes up-regulated by saponin were identified as up-regulated by host cell contact (Table 1).

RpoH is induced upon contact with A431 cells. Among the genes identified as up-regulated following adherence to epithelial cells is *rpoH*, which encodes the putative heat shock sigma factor, σ^{32} (RpoH). In *E. coli*, RpoH has been shown to regulate the expression of genes encoding a number of molecular chaperones and other proteins that are important for keeping proteins in the cell folded properly under conditions of stress, such as increased temperature (24). Two major genes known to be transcribed from σ^{32}-dependent promoters in gram-negative bacteria are *groEL* and *groES*, which encode such molecular chaperones. Not surprisingly, our results showed the gonococcal *groEL* and *groES* homologs to be up-regulated in gonococci upon cell contact (Table 1). This is consistent with a previous report showing that transcription of the gonococcal *groESL* operon is increased following heat shock (79).

RT-PCR analysis of gonococcal genes induced upon host cell contact. In order to corroborate the DNA microarray results, quantitative RT-PCR was employed to measure differential expression of *rpoH* and *groES*. 16S rRNA expression was used as an internal control, since its expression was not expected to be influenced by bacterium-host cell contact. *N. gonorrhoeae* strain MS11 was used to infect A431 cells at an MOI of 10. At 3 h p.i., RNA was isolated from adherent bacteria and from bacteria grown in cell culture medium in the absence of cells, essentially as described for the DNA array

TABLE 1. Genes up-regulated in gonococci adherent to A431 cells

ORF ID[a]	Gene[a]	Gene product	Expression ratio[b]	(SD)
NG1684		Conserved hypothetical protein	3.27	(1.33)
NG0288	*rpoH*	RpoH, σ^{32}	2.61	(0.61)
NG1363	*mtrE*	MtrE, multidrug efflux pump	2.48	(0.88)
NG2094	*groES*	GroES, heat shock chaperone	2.39	(0.42)
NG1989		*Neisseria*-specific protein, uncharacterized	2.38	(0.67)
NG0340	*cysK*	Cysteine synthase	2.34	(0.95)
NG0238		Conserved hypothetical protein	2.34	(1.10)
NG1210		Conserved hypothetical protein	2.24	(0.50)
NG2095	*groEL*	GroEL, heat shock chaperone	2.10	(0.24)
NG0634		Hypothetical protein	2.03	(0.72)
NG0372		ABC transporter	1.97	(0.41)
NG0791		Conserved hypothetical protein	1.93	(0.71)
NG1927		*Neisseria*-specific protein uncharacterized	1.91	(0.43)
NG0635		Hypothetical protein	1.91	(0.51)
NG1083		Hypothetical protein	1.89	(0.50)
NG0633	*nifU*	Fe-S scaffold protein	1.89	(0.78)
NG1988		Conserved hypothetical protein	1.88	(0.48)
NG0234	*hemN*	Porphyrin oxidoreductase	1.88	(0.63)
NG0376	*ppiB*	Peptidyl-prolyl *cis-trans* isomerase B	1.87	(0.86)
NG0721		*Neisseria*-specific protein	1.85	(0.38)
NG1366	*mtrR*	MtrR, multidrug efflux regulator	1.84	(0.65)
NG1291		Conserved hypothetical protein	1.83	(0.25)
NG0199	*rho*	Transcription termination factor Rho	1.79	(0.62)
NG1515	*tyrC*	Prephenate dehydrogenase TyrC	1.76	(0.11)
NG1493	*rpsT*	30S ribosomal protein S20	1.73	(0.36)
NG1439	*aqpZ*	ABC transporter, ATP binding	1.72	(0.34)
NG0989		Conserved hypothetical protein	1.69	(0.44)
NG1831	*rpmC*	50S ribosomal protein L29	1.68	(0.61)
NG1841	*rspJ*	30S ribosomal protein S10	1.68	(0.24)
NG1668	*pgi*	Glucose-6-phosphate isomerase	1.67	(0.38)
NG0637		Conserved hypothetical protein	1.67	(0.18)
NG1773	*sdaA*	L-serine dehydratase; L-serine deaminase	1.65	(0.35)
NG2180		Conserved hypothetical protein	1.59	(0.56)
NG0962	*pncB*	Nicotinate phosphoribosyltransferase	1.59	(0.31)
NG5041	*ydhB*	Hypothetical protein, MS11 island	1.46	(0.37)
NG1587	*mafB2*	Adhesin MafB2	1.41	(0.39)
NG0276	*comA*	Competence protein (ComA)	1.32	(0.30)

[a] ORF identification numbers and gene designations are from the annotated *N. gonorrhoeae* genome sequence (8).
[b] Ratio of expression in adherent bacteria to expression in bacteria grown in the absence of epithelial cells.

experiments. RNA was quantified and subjected to RT-PCR using primers specific for *rpoH*, *groES*, and 16S rRNA. Figure 1A shows the results of a representative RT-PCR experiment. These data show that expression of *rpoH* was greatly increased (4.32-fold) in wild-type gonococci (MS11) at 3 h p.i. Expression of *groES* was also significantly increased in MS11 (1.77-fold), while the expression of 16S rRNA was unchanged. These data are consistent with the results of the DNA microarray experiments.

An additional control was done to rule out effects of saponin (used to lift cells with adherent bacteria). Gonococci grown on plates were swabbed into GC broth and divided into two aliquots: the first into GC broth, the second into GC broth containing 1% saponin. After 10 min of incubation, RNA was isolated from both samples and subjected to RT-PCR using *rpoH*-specific primers. Results of this experiment showed that *rpoH* expression was not affected by treatment of the bacteria with saponin (data not shown).

RpoE does not mediate the induction of *rpoH* in gonococci upon host cell contact. In *E. coli*, the expression of *rpoH* is under the control of multiple promoters, three that are transcribed under most growth conditions via the housekeeping

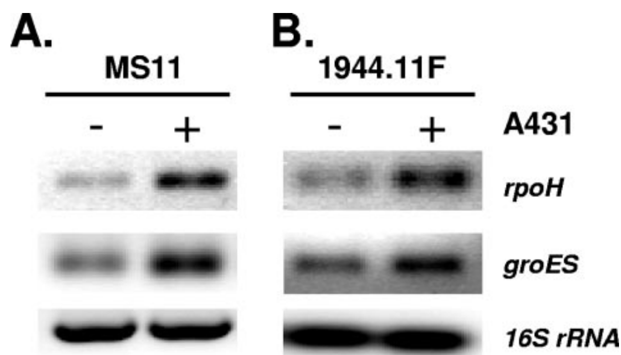

FIG. 1. RT-PCR analysis of genes induced in gonococci adherent to human epithelial cells. RNA isolated from MS11 (wild-type) or NG1944.11F (*rpoE*::TnEmUP) cells adherent to A431 cells (+) or grown in the absence of A431 cells (-) was reverse transcribed using random primers. The subsequent PCR was performed with primers specific for *rpoH*, *groES*, and 16S rRNA, as indicated on the right. Images of EtBr-stained gels were reversed using Adobe Photoshop for clarity, and band intensities were quantified using Kodak image analysis software, normalizing to 16S rRNA. Results shown are representative of three independent determinations.

sigma factor, σ^{70} (24), one of which is subject to catabolite repression (54). The fourth promoter is dependent on the extreme heat shock sigma factor, σ^{E} (encoded by *rpoE*), which responds to misfolded extracytoplasmic proteins (64). It is thought that RpoE is important for the maintenance of membrane and periplasm homeostasis, and it has been shown to have a role in pathogenesis in several gram-negative bacteria (10, 38, 80).

Since *N. gonorrhoeae* has an annotated gene (NG1944) with high similarity to *rpoE* (8), we initially hypothesized that the observed response to host cell contact might be mediated by RpoE-dependent induction of *rpoH*, which in turn would lead to RpoH-dependent induction of *groEL*/*groES*. To test this hypothesis, an *rpoE* mutant of *N. gonorrhoeae* strain MS11 was constructed. The gonococcal *rpoE* gene was PCR amplified from *N. gonorrhoeae* genomic DNA and cloned into pDONR221, and the resulting plasmid (pNG1944) was mutagenized by in vitro transposition using the transposon TnErmUP as described elsewhere (16). Two plasmids containing *rpoE* with transposon insertions approximately 320 bp (11F) and 500 bp (9G) from the *rpoE* start codon were isolated and used to transform *N. gonorrhoeae* strain MS11. Em^r transformants were obtained at a high frequency ($\sim 1 \times 10^{-5}$ Em^r transformants/CFU), indicating that *rpoE* is not essential in *N. gonorrhoeae*. This is inconsistent with observations that *rpoE* is essential in several bacteria, including *E. coli* and *Yersinia enterocolitica* (12, 29). To rule out the possibility that the Em^r transformants were *rpoE* heterodiploids, having a copy of the intact *rpoE* gene in addition to the insertionally inactivated copy, PCR was done using primers specific for *rpoE*. Agarose gel analysis of the amplification products showed that the mutants yielded a band ~1.2 kb larger than the wild-type *rpoE*, consistent with the insertion of the TnErmUP transposon (data not shown). None of the mutants yielded a band corresponding to the size of the wild-type *rpoE* gene. Expression of *rpoE* in the mutants was analyzed by RT-PCR, with no amplification products observed except in the wild-type (MS11) control. Hence, our results indicate that strains NG1944.11F and NG1944.9G are indeed *rpoE* null mutants and that *rpoE* is not essential to *N. gonorrhoeae*.

To examine the role of RpoE in the initial stages of a gonococcal infection, adhesion and invasion assays were done using A431 cells as described in Materials and Methods. Adhesion was scored as the number of cell-associated CFU/total CFU at the end of the experiment, while invasion was scored as the number of bacteria protected from gentamicin killing (Gm^r)/number of cell-associated CFU. The results of these assays are summarized in Fig. 2. The *rpoE* mutant (NG1944.11F) adhered to and invaded A431 cells at essentially the same frequencies as the wild-type parent strain, MS11 ($P >$ 0.05, Student's t test). These results suggest that RpoE is not necessary for gonococcal adherence to or invasion of A431 cells.

To determine whether *rpoH* expression and induction upon adherence to epithelial cells was dependent on RpoE, RT-PCR was done using RNA isolated from NG1944.11F grown either alone in cell culture medium or following adherence to A431 cells for 3 h. The results of these experiments (shown in Fig. 1B) showed that *rpoH* is up-regulated in the *rpoE* mutant at 3 h p.i., similar to what was observed in the wild-type strain,

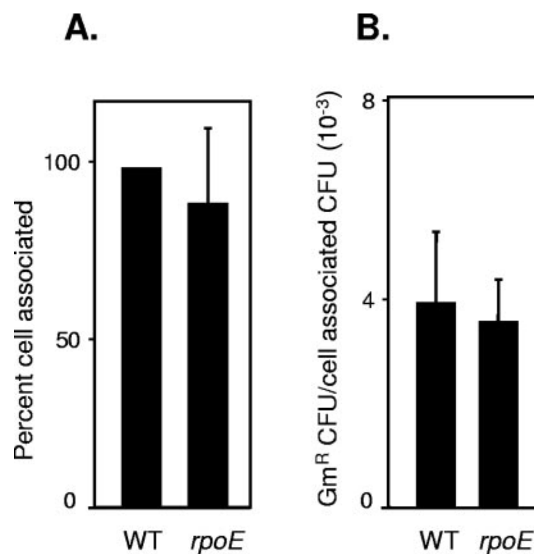

FIG. 2. Adherence to and invasion of A431 cells are not affected in a gonococcal *rpoE* mutant. WT, MS11; *rpoE*, *rpoE*::TnEmUP. (A) Adherence to A431 cells. Adhesion data are presented as a percentage of the wild-type control (MS11) and are averages of five independent experiments performed in triplicate. (B) Invasion of A431 cells. Invasion data are presented as the ratio of gentamicin-resistant (Gm^r) bacteria per number of cell-associated bacteria and are averages of two independent experiments performed in triplicate. Error bars indicate standard deviations.

MS11 (Fig. 1A). Expression of *groES* followed a similar pattern. These data suggest that *rpoE* is not required for the up-regulation of *rpoH* or *groES* upon host cell contact.

To analyze global gene expression in *rpoE* mutant gonococci, a DNA microarray experiment was performed. Labeled cDNA was synthesized from RNA isolated from NG1944.11F (*rpoE*::TnEmUP) following 3-h adherence to A431 cells as described above for wild-type gonococci. Analysis of the data showed a similar list of genes up-regulated by host cell contact as was observed for the wild-type parent, MS11, including *rpoH*, *groEL*, and *groES* (data not shown). Taken together, our results indicate that RpoE is not involved in regulating *rpoH* gene expression in gonococci, at least under the conditions tested.

RpoH is essential to *N. gonorrhoeae*. In order to examine the role of RpoH in gonococci and its interaction with host cells, we next attempted to construct an *rpoH* null mutant. The gonococcal *rpoH* gene was PCR amplified and cloned into pHSS6, and the resulting plasmid (pADC1) was mutagenized with the transposon TnErmUP as described above for *rpoE*. Three different mutants with transposon insertions of approximately 220 bp (1H), 435 bp (5D), and 700 bp (6G) from the *rpoH* start codon were used to transform *N. gonorrhoeae* strain MS11. Despite several attempts at transformation with increasing amounts of input DNA, Em^r mutants were never isolated. Positive transformation controls included plasmid DNA used to construct the *rpoE* mutants (described above), as well as genomic DNA from a previously characterized Em^r *N. gonorrhoeae* strain, MS11-306 (49). The inability to obtain Em^r *rpoH* null mutants strongly suggested that *rpoH* is essential in *N. gonorrhoeae*. An alternative explanation for our result could be

a polar effect on expression of a gene downstream (3′) of *rpoH*; however, this is unlikely, as the gene immediately 3′ of *rpoH* (NG0287) is transcribed in the opposite direction (8). Hence, we concluded from these results that *rpoH* is essential to gonococci.

Construction of a conditional *rpoH* mutant. Since we were unable to construct an *rpoH* null mutant in *N. gonorrhoeae*, a strain was constructed in which *rpoH* expression was under the transcriptional control of the lactose repressor (LacI) such that *rpoH* expression could be controlled by the addition of the LacI inducer isopropyl-β-D-thiogalactopyranoside (IPTG) to the culture medium. We utilized a plasmid, pKH35 (27), which contains a segment of the *N. gonorrhoeae* chromosome (3.8 kb) in which large sequences can be inserted without being deleterious to the cell (H. S. Seifert, personal communication). Between the *aspC* and *lctP* genes on the plasmid is a selectable marker (Cm^r), a *lacI*^{q} gene (since *N. gonorrhoeae* has no *lac* operon of its own), two tandem *lac* promoter-operator sequences (*lacOPOP* [70]), and three *Neisseria* uptake sequences, which are necessary for gonococcal transformation (21). A gene of interest cloned downstream of *lacOPOP* and transformed into *N. gonorrhoeae* results in recombination between the incoming DNA and the *aspC-lctP* region of the chromosome. Since pKH35 can replicate in *E. coli* but not *N. gonorrhoeae*, the plasmid itself is lost following transformation. What remains on the chromosome is the selectable marker and the IPTG-inducible construct.

The *rpoH* gene was PCR amplified from MS11 genomic DNA and cloned immediately downstream of the *lac* promoter on pKH35. This plasmid, pNGR18, was then used to transform MS11, selecting for Cm^r. A double crossover event following transformation results in the insertion of the inducible *rpoH* between *aspC* and *lctP*. The resulting strain, MP288, has two copies of *rpoH* on the genome, the native copy and the inducible copy. To eliminate the native copy of *rpoH* (such that *rpoH* expression is solely under IPTG control), MP288 was transformed with the *rpoH*::TnErmUP plasmid, pNGR20.5D, and transformants were selected for Em^r on plates containing IPTG, to ensure some *rpoH* expression in the event the native copy of *rpoH* was inactivated, as intended. Since this incoming DNA could recombine with either the native copy of *rpoH* or the inducible *rpoH* locus, Em^r transformants were screened by PCR using primers that differentiated between the native *rpoH* locus and the inducible construct at the *lctP-aspC* locus. A mutant was identified in which only the wild-type copy of the *rpoH* gene was insertionally inactivated, which was named MPD288 (*rpoH*::TnEmUP *lacIOP-rpoH*Cm) and expresses *rpoH* only in the presence of IPTG. MPD288 was maintained on GC agar plates containing 20 μM IPTG, which allowed growth similar to the wild-type strain. Subsequent passage of this strain onto medium lacking IPTG resulted in an eventual growth defect, but only after several passages. This may be due to a small amount of RpoH still existing in the cell and/or leaky expression of *rpoH* from the *lacOPOP* promoter.

The expression and inducibility of *rpoH* in strain MPD288 was examined by RT-PCR using RNA isolated from bacteria grown with and without IPTG. Figure 3 shows that *rpoH* is expressed at barely detectable levels in the absence of IPTG in MPD288 but at nearly wild-type levels in the presence of 20 μM IPTG. Increasing the IPTG resulted in corresponding increases in *rpoH* mRNA (data not shown).

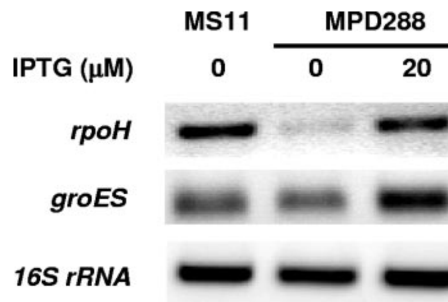

FIG. 3. RT-PCR of *rpoH* and *groES* in an *N. gonorrhoeae* strain conditionally expressing *rpoH*. RNA isolated from MS11 (wild type) or MPD288 (*rpoH*::TnEmUP *lacIOP-rpoH*Cm) grown overnight on GC agar with or without 20 μM IPTG (as indicated) was reverse transcribed using random primers. The subsequent PCR was performed with primers specific for *rpoH*, *groES*, and 16S rRNA, as indicated on the left. Images of EtBr-stained gels were reversed using Adobe Photoshop for clarity, and band intensities were quantified using Kodak image analysis software, normalizing to 16S rRNA. Results shown are representative of three independent determinations.

Since *groES*, which is also induced in gonococci upon adherence to A431 cells (Table 1 and Fig. 1), is expected to be dependent on RpoH, we also analyzed expression of this gene in the conditional *rpoH* strain. As predicted, expression of *groES*, but not 16S rRNA, was reduced in the conditional *rpoH* strain grown under *rpoH* depletion conditions (no IPTG) (Fig. 3), confirming that *groES* expression in gonococci is dependent on RpoH.

Temperature sensitivity of a conditional *rpoH* strain. In gram-negative bacteria, the RpoH regulon is induced in response to a sudden increase in temperature, as well as other stresses. This response is necessary to maintain the proper folding of proteins in the cytoplasm under such conditions which could otherwise be lethal to the cell. An inability to respond to heat shock (as is mediated by RpoH) would likely make the bacterium more sensitive to heat shock. To test this in gonococci, we asked whether depletion of RpoH, using our conditional *rpoH* strain, MPD288, would affect the strains' ability to survive heat shock. *N. gonorrhoeae* strains MS11 (wild-type) and MPD288 (inducible *rpoH*) grown on GC plates with or without 20 μM IPTG were swabbed into GC broth and diluted to 10^7 CFU/ml. The bacterial suspensions were then subjected to heat shock at 42°C for various times over a 1-h period. At 10-min intervals, samples were collected and plated to determine viable CFU. The data obtained from four independent experiments were averaged and are plotted in Fig. 4. Gonococci depleted for RpoH (MPD288 grown without IPTG) were more sensitive to heat shock than the wild-type strain (MS11) or the conditional *rpoH* strain grown in the presence of IPTG. This result indicates that *rpoH* expression contributes to gonococcal survival in response to heat shock.

Effect of RpoH on interactions between gonococci and A431 cells. DNA array analysis of gene expression in gonococci adherent to A431 cells showed that *rpoH* is up-regulated more than twofold at 3 h p.i. (Table 1). Genes expected to be dependent on RpoH for expression, such as *groES* and *groEL*, are also significantly up-regulated under these conditions. These

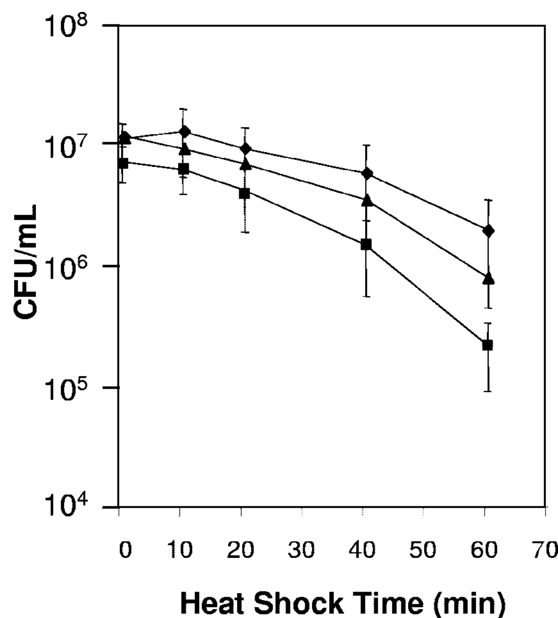

FIG. 4. *N. gonorrhoeae* cells depleted of RpoH are hypersensitive to heat shock. Bacteria were grown overnight on GC agar plates with or without 20 μM IPTG (as indicated) and then swabbed into GC broth at 10^7 CFU/ml. Bacterial suspensions were subjected to a heat pulse at 42°C, and aliquots were removed at 10-min intervals. Serial dilutions of samples were plated on GC agar plates (plus 20 μM IPTG as necessary). Symbols: (◆), MS11 (wild type); ▲, MDP288 (inducible *rpoH*) plus 20 μM IPTG; ■, MDP288 (inducible *rpoH*) without IPTG. Error bars indicate standard deviations.

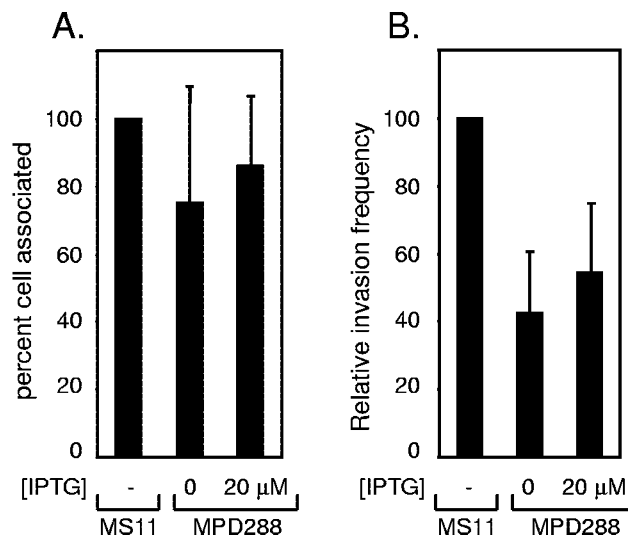

FIG. 5. Depletion of RpoH in *N. gonorrhoeae* affects invasion of, but not adherence, to A431 cells. MS11, wild type; MDP288, *rpoH*::TnEmUP *lacIOP-rpoH*Cm. (A) Adherence to A431 cells. Adhesion data are presented as a percentage of the wild-type control (MS11) and are averages of four independent experiments performed in triplicate. (B) Invasion of A431 cells. Invasion data are presented as relative invasion frequency, normalized to MS11. These data are averages of five independent experiments performed in triplicate. Error bars indicate standard deviations.

results imply that RpoH and genes it regulates might be important for subsequent steps in a gonococcal infection. Thus, we next asked whether *rpoH* expression is necessary for gonococci to adhere to and invade A431 cells in culture.

Wild-type *N. gonorrhoeae* strain MS11 and MPD288 were used to infect A431 cells at an MOI of 10. To deplete RpoH in MPD288, this strain was maintained on GC agar containing 20 μM IPTG and then grown overnight on medium lacking IPTG prior to infection of A431 cells. For infection, bacteria were harvested from plates and a suspension prepared in cell culture medium with or without IPTG. At the end of the experiment, samples were plated on GC agar containing 20 μM IPTG and CFU were enumerated following incubation at 37°C.

The results of these experiments are summarized in Fig. 5. Adhesion frequencies, determined at 3 h p.i., showed no significant differences between MPD288 grown with or without IPTG and the wild-type parent strain, MS11 ($P > 0.05$). However, the subsequent step in infection, invasion of epithelial cells, appeared to be reduced for the mutant MPD288 depleted for RpoH. The relative invasion frequency at 7 h p.i. for this strain was significantly less than that observed for the wild-type strain, MS11 ($P = 0.002$). MPD288 grown under conditions which led to expression levels of *rpoH* comparable to the wild type (Fig. 3) invaded A431 cells at a higher frequency than this strain grown without IPTG, although the frequency was not as high as observed for the wild-type parent and may not be significantly different ($P = 0.06$). The reduced ability of the *rpoH*-expressing MPD288 strain to invade A431 cells compared to MS11 may be due to an inability of this strain to induce *rpoH* expression upon adherence, since the native regulatory sequences are not present upstream of the expressed *rpoH* gene in this strain. Taken together, these data show that in wild-type gonococci, *rpoH* expression is turned on in response to initial contact with epithelial cells, and this expression is necessary for the subsequent invasion of these cells. This demonstrates a mechanism for this bacterium to sense its environment and adjust gene expression in preparation for the next step of the infection.

DISCUSSION

The interaction between a host and a pathogen is a dynamic process, involving responses from both the pathogen and the host. Several bacterial pathogens respond to changes in their environment by modulating gene expression, providing a selective advantage to organisms that are, at least initially, outnumbered, and must deal with differences in nutrient availability as well as with the (often-hostile) response of the host. Often, these regulatory systems result in the integration of responses to several signals via complex networks to modulate the expression of a variety of genes that are important during an infection (15, 23, 45, 51, 66, 75). Some bacteria, such as *Yersinia pseudotuberculosis* (60) and uropathogenic *E. coli* (86), regulate gene expression specifically in response to contact with host cells. Since one of the first events to occur when the *N. gonorrhoeae* enters a new host is adherence to the mucosal epithelia, we hypothesized that this might serve as a signal for the bacterium to modulate the expression of genes that will allow it to colonize and proliferate in the new host. It has been shown that adherence of gonococci to epithelial cells in culture enhances their invasiveness (11), and it is likely that this in-

TABLE 2. Genes down-regulated in gonococci adherent to A431 cells

ORF ID[a]	Gene[a]	Gene product	Expression ratio[b]	(SD)
NG1024		Conserved hypothetical protein	5.44	(1.26)
NG0186	*ald*	Zn-alcohol dehydrogenase	2.55	(1.24)
NG0715	*g6pd*	Glucose 6-phosphate 1-dehydrogenase	2.54	(0.25)
NG0186.1		Transposase fragment of IS1016	2.54	(1.57)
NG0574	*cah*	Carbonic anhydrase	2.52	(0.72)
NG0771	*recD*	RecD	2.35	(0.26)
NG1215		Conserved hypothetical protein	2.24	(0.70)
NG1358	*gdhA*	Glutamate dehydrogenase	2.04	(0.50)
NG1473	*mdaB*	Drug activity modulator B	1.99	(0.26)
NG1882		Hypothetical protein	1.96	(0.79)
NG1600	*glnA*	Glutamine synthetase	1.92	(0.44)
NG1545		Conserved hypothetical protein	1.89	(0.56)
NG1627		Hypothetical protein	1.85	(0.53)
NG0656	*oxiT*	Oxalate/formate antiporter	1.84	(0.89)
NG1630		Lambda repressor-like protein *cI*	1.83	(0.85)
NG1698	*comE*	Competence protein ComE	1.78	(0.44)
NG1139		*Neisseria*-specific protein	1.76	(0.70)
NG0479		Phage-related repressor (*cI*)	1.76	(0.26)
NG0849	*proB*	ProB	1.73	(0.57)
NG1249		Conserved hypothetical protein	1.71	(0.45)
NG1117		Conserved hypothetical protein (phage-like)	1.71	(0.68)
NG0249	*accD*	Acetyl coenzyme A carboxylase	1.70	(0.20)
NG1881	*pykA*	Pyruvate kinase II	1.70	(0.64)
NG0177	*cpxR*	CpxR	1.70	(0.62)
NG0850	*proA*	ProA	1.69	(0.33)
NG1769	*yhjA*	Cytochrome *c* peroxidase	1.68	(0.37)
NG0788		*Neisseria*-specific protein, uncharacterized	1.67	(0.29)
NG1258	*pgm*	Phosphoglycerate mutase	1.67	(0.20)
NG0561		Conserved hypothetical protein	1.67	(0.33)
NG1406	*gcsT*	Aminomethyltransferase, glycine cleavage system	1.66	(0.40)
NG1815	*minD*	Cell division protein, MinD	1.66	(0.44)
NG0904		Conserved hypothetical protein	1.66	(0.40)
NG0062	*fthS*	Formate-tetrahydrofolate ligase	1.65	(0.46)
NG0959		Conserved hypothetical protein	1.65	(0.35)
NG0719	*g6pi*	Glucose-6-phosphate isomerase	1.65	(0.26)
NG0794	*bfrA*	Bacterioferritin A	1.64	(0.58)
NG0410	*cspA*	Cold shock protein	1.64	(0.14)
NG1122		Hypothetical protein	1.64	(0.37)
NG0318	*recN*	RecN	1.64	(0.50)
NG0449		Conserved hypothetical protein	1.63	(0.50)
NG0717	*hxkG*	Glucokinase	1.63	(0.40)
NG1577	*omp3*	PIII	1.63	(0.37)
NG0732		*Neisseria*-specific protein, uncharacterized	1.62	(0.59)
NG0626	*mltB*	Murein transglycosylase	1.62	(0.16)
NG0692		LysR family transcriptional regulator	1.62	(0.34)
NG0472		Hypothetical protein	1.61	(0.42)
NG1300	*yfcB*	Adenine-specific methylase	1.61	(0.29)
NG0787		*Neisseria*-specific protein, uncharacterized	1.59	(0.25)
NG1374	*ccoN*	Cytochrome *c* oxidase	1.59	(0.31)
NG1643		Conserved hypothetical protein	1.58	(0.14)
NG1138		Conserved hypothetical protein	1.57	(0.19)
NG0248	*trpA*	Tryptophan synthase alpha chain	1.56	(0.22)
NG0203	*gph*	Phosphoglycolate phosphatase	1.55	(0.30)
NG1110	*dnaB*	DnaB	1.55	(0.42)
NG0716	*6pgl*	6-Phosphogluconolactonase	1.54	(0.37)
NG1381	*glr2*	Glutaredoxin 2	1.51	(0.41)
NG1411		Conserved hypothetical protein	1.40	(0.66)

[a] ORF identification numbers and gene designations are from the annotated *N. gonorrhoeae* genome sequence (8).
[b] Ratio of expression in bacteria grown in the absence of epithelial cells to expression in adherent bacteria.

creased invasiveness results from changes in gene expression that occur in response to adherence to cells. Thus, in this work, we sought to identify the genes that are differentially expressed in gonococci in response to attachment to epithelial cells and to identify the regulatory networks involved.

A PCR amplicon-based genome microarray was used to examine changes in gene expression in *N. gonorrhoeae* upon adherence to human epithelial cells. The results of these experiments showed 37 genes were up-regulated (Table 1) and 57 genes were down-regulated (Table 2) in adherent gonococci

relative to bacteria grown in the absence of host cells. These genes fall into several categories, including transcriptional regulation, bacterial metabolism, molecular transport, and (as expected) several hypothetical genes of unknown function.

Of the genes down-regulated upon adherence, several of them (22/57) encode proteins involved in central housekeeping functions, such as glycolysis, amino acid metabolism, nitrogen assimilation, respiration, and cell division. This suggests that there might be a general shift in cellular metabolism in response to host cell contact, indicating a mechanism for the bacterium to begin to adapt to different nutrient availability in the new host and/or the different environment inside of the host cell. Other down-regulated genes encode proteins involved in DNA replication, recombination, and repair (RecD, RecN, DnaB, and YfcB), transformation competence (ComE), and four potential transcriptional regulators. Interestingly, one of the down-regulated genes, NG1473, encodes MdaB, a protein thought to be a drug activity modulator (8). However, MdaB has recently been shown to be an NADPH quinone reductase that plays an important role in managing oxidative stress in bacteria, suggestive of a role in pathogenesis (83).

In addition to the hypothetical genes up-regulated upon adherence (16/37) were several genes encoding proteins involved in cellular metabolism and general gene expression, again suggesting a general shift in bacterial metabolism in response to host cell contact. NG1587, which was slightly up-regulated on adherence, is annotated as *mafB2*, one of several genes of the *m*ultiple *a*dhesin *f*amily (*maf*), which encode glycolipid adhesins (57). This up-regulation of *mafB2* could indicate a need for MafB2 at subsequent steps of an infection, consistent with reports that MafB2 is involved in Opa-independent invasion (57).

Of particular interest was the observation that several putative transcriptional regulators were differentially regulated upon host cell contact, two up-regulated (Table 1) and four down-regulated (Table 2). The identification of multiple putative transcriptional regulators as repressed as well as induced upon adherence to epithelial cells suggests that there are indeed complex regulatory networks involved in the modulation of gene expression in gonococci in response to host cell contact. Interestingly, NG0177, which is annotated as *cpxR* (8), is down-regulated upon host cell contact. In *E. coli*, CpxR is a regulator that responds to cell envelope stress, in particular to misfolded proteins at the inner membrane (reviewed in reference 63). However, NG0177 is also annotated at *ompR* in *N. gonorrhoeae*, *phoQ* in *N. meningitidis* serogroup B (32), and *misR* in *N. meningitidis* serogroup A (81). Thus, the function of the protein encoded by NG0177 remains to be determined.

One of the highest up-regulated genes identified in our experiments was *rpoH*, which encodes a heat shock-specific sigma factor (RpoH, σ^{32}). In gram-negative bacteria, σ^{32} up-regulates the expression of genes encoding a number of proteins (particularly chaperones and proteases) that are necessary for keeping proteins in the cell folded properly under conditions of stress, such as increased temperature (reviewed in references 24 and 85). *groEL* and *groES*, which encode such molecular chaperones and are known to be transcribed from σ^{32}-dependent promoters in many gram-negative bacteria, were also identified as up-regulated in gonococci upon adherence to host cells (Table 1; Fig. 1). Our initial hypothesis was that the up-regulation of gonococcal *rpoH* and the RpoH regulon would be dependent on the extracytoplasmic sigma factor σ^E (encoded by *rpoE*). σ^E is known to control *rpoH* expression in *E. coli* and *Salmonella enterica* serovar Typhimurium in response to misfolded extracytoplasmic proteins (52, 64). Our rationale was that an interaction between host cells and the outer surface of the gonococcus might be a signal to which RpoE would respond and subsequently up-regulate *rpoH* expression. Construction and analysis of a gonococcal strain with a transposon insertion in *rpoE*, however, showed that RpoE was not necessary for the induction of *rpoH* upon host cell contact (Fig. 1). This may be due in part to the fact that there are no apparent gonococcal homologs to the anti-sigma factor, RseA, and its accessory protein, RseB, which serve to control RpoE activity in response to extracytoplasmic signals (12).

While it is clear that RpoE does not control *rpoH* gene expression in gonococci in response to host cell contact, the regulatory mechanism used by gonococci to up-regulate *rpoH* gene expression in response to host cell contact is still to be determined. It is possible that transcriptional control of *rpoH* expression in gonococci is mediated by other regulators in response to host cell contact, as yet to be identified. Clearly, expression of *rpoH* is important in gonococci, since we (and others [42]) have been unable to construct an *rpoH* null mutant, suggesting that it is an essential gene.

Control of RpoH activity in gram-negative bacteria is complex and occurs only partially at the level of *rpoH* transcription (24, 85). Transcription of *rpoH* is usually high under steady-state conditions but the message is not efficiently translated until a shift in growth temperature, which results in an increase in translation of the preexisting *rpoH* mRNA by destabilization of a secondary structure in the mRNA which overlaps the *rpoH* start codon (53). In addition, RpoH activity is controlled by two RpoH-dependent chaperone systems, DnaK/J and GroEL/S, which can sequester σ^{32}, preventing its association with RNA polymerase holoenzyme (26, 44). Thus, the heat shock response is modulated by the protein folding status of the cell.

A recent report suggests that regulation of RpoH in *N. gonorrhoeae* may differ from that observed in other bacteria (42). While an increase in *rpoH* expression was observed upon heat shock, the time following heat shock that the increase was observed was less than the time observed for induction of the RpoH-dependent genes, *dnaJ*, *dnaK*, and *grpE*. The level of RpoH protein, as shown by immunoblotting with antisera raised against *E. coli* RpoH, correlated with *rpoH* expression, which the report's authors concluded ruled out translational regulation, as has been demonstrated for *E. coli rpoH* (53). The conclusion of this work was that regulation of RpoH activity is the primary level of regulation in response to heat shock in gonococci.

Our observation that *rpoH* is induced upon host cell contact in gonococci is a determination of the relative levels of *rpoH* transcript at a single point in time (3 h p.i.). The increase in *rpoH* transcript levels in adherent bacteria relative to that in nonadherent bacteria could be due to increased transcription or to an increase in *rpoH* mRNA stability, which cannot be distinguished from one another in these experiments. It is clear from our results, however, that the interaction between gonococci and epithelial cells serves to transmit a signal to the

bacterium, by an as-yet-undefined mechanism, that results in the induction of *rpoH* and at least some genes of the RpoH regulon. Regulation of RpoH activity was not directly assessed and could, in part, play a role in the induction of *groEL*, *groES*, and other genes upon host cell contact. Further experiments are required to examine this.

The observation that *rpoH*, as well as two genes of the RpoH regulon, *groES* and *groEL*, but not *dnaJ*, *dnaK*, and *grpE*, which are known to be RpoH dependent in gonococci (42), were up-regulated in our studies could indicate the involvement of multiple regulatory systems, as yet unidentified. RpoH may respond to more than heat shock in the gonococcus. As a strict human pathogen with no environmental niche outside its host, *N. gonorrhoeae* is likely to encounter only slight temperature fluctuations but numerous other environmental stresses in an infection or upon transmission. In other bacteria, RpoH responds to stresses such as increased temperature as well as other factors, such as increased hydrostatic pressure and hyperosmotic shock (1, 6), enabling the bacterium to detect and correct misfolded proteins in the cytoplasm either by chaperone-assisted folding or degradation by proteases (24).

Bacterial pathogens often up-regulate the production of virulence factors upon infection, many of which are directly involved in host interactions and are on the surface of the bacterium. These proteins are often large, complex, multisubunit proteins that require the assistance of chaperones and other proteins in the bacterial cell to assemble correctly and/or to be exported to their final destination. If the assembly and export functions are not adequate, accumulation of misfolded proteins in the cytoplasm would need to be degraded by proteases. An increase in the production of chaperones to aid in the proper folding and assembly of virulence factors concomitant with an increase in their expression would be critical in the adaptation process.

Our results demonstrating a role for the heat shock response in gonococcal pathogenesis suggest that bacterial heat shock regulons might have a significant role in the regulation of virulence. Another example is *Vibrio cholerae*, in which the expression of several virulence genes including the cholera toxin gene (*ctx*) are modulated by growth temperature as well as other environmental factors reviewed in references (39 and 59).

A final interesting note is that while many bacteria use several sigma factors, even regulatory cascades of sigma factors, to control gene expression in response to a variety of environmental conditions, *N. gonorrhoeae* only appears to encode four sigma factors: σ^E (RpoE), σ^{32} (RpoH), σ^{70} (RpoD), and σ^{54} (RpoN). σ^{70} (RpoD) is the primary "housekeeping" sigma factor, highly conserved among gram-negative bacteria (25). In *N. gonorrhoeae*, σ^{54} (RpoN) has been shown to be nonfunctional (41). As we have shown in this work, σ^{32} (RpoH), but not σ^E (RpoE), is essential for viability of gonococci and also plays a role in interactions between gonococci and the host. However, σ^E (RpoE) does not appear to play a role in controlling expression of *rpoH*, at least under the conditions tested, and preliminary experiments in this laboratory have yet to identify a role for RpoE in gonococcal gene expression. In addition, gonococci do not appear to have a homolog of the general stress sigma factor, σ^s, that in many gram-negative bacteria regulates gene expression in response to stresses such as starvation, hyperosmolarity, pH downshift, or nonoptimal temperature (reviewed in reference 28).

In summary, we have examined global gene expression in gonococci in response to adherence to epithelial cells in culture. The results of our array experiments indicate that the interaction between gonococci and the host results in changes in transcription of several genes which can lead to changes in bacterial cell metabolism and other basic functions, enabling the bacterium to quickly adapt to its new environment.

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