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RpoH Mediates the Expression of Some, but Not All, Genes Induced in *Neisseria gonorrhoeae* Adherent to Epithelial Cells

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***Neisseria gonorrhoeae* (gonococcus [GC]), is highly adapted to the human host, the only known reservoir for gonococcal infection. However, since it is sexually transmitted, infection of a new host likely requires a regulatory response on the part of the gonococcus to respond to this significant change in environment. We previously showed that adherence of gonococci to epithelial cells results in changes of gene expression in the bacteria that presumably prepare them for subsequent steps in the infection process. Expression of the heat shock sigma factor gene, *rpoH*, was shown to be important for the invasion step, as gonococci depleted for *rpoH* were reduced in their ability to invade epithelial cells. Here, we show that of the genes induced in adherent gonococci, two are part of the gonococcal RpoH regulon. When RpoH is depleted, expression of these genes is no longer induced by host cell contact, indicating that RpoH is mediating the host cell induction response of these genes. One RpoH-dependent gene, NGO0376, is shown to be important for invasion of epithelial cells, consistent with earlier observations that RpoH is necessary for this step of infection. Two genes, NGO1684 and NGO0340, while greatly induced by host cell contact, were found to be RpoH independent, indicating that more than one regulator is involved in the response to host cell contact. Furthermore, NGO0340, but not NGO1684, was shown to be important for both adherence and invasion of epithelial cells, suggesting a complex regulatory network in the response of gonococci to contact with host cells.**

Neisseria gonorrhoeae (gonococcus) is a gram-negative diplococcus which causes the sexually transmitted disease, gonorrhea. It is an obligate human pathogen which typically infects the urethra in men and endocervix in women, although it occasionally infects other tissues as well. Gonorrhea is a continuing threat to public health worldwide, especially in developing countries and some lower socioeconomic groups in developed countries. According to the World Health Organization (www.who.org), about 30 million incidents of gonococcal disease occur each year, a figure that is likely underestimated due to the large reservoir of asymptomatic carriers (70). Another serious problem, in light of the global AIDS epidemic, is the finding that gonorrhea can significantly enhance the transmission of human immunodeficiency virus (39, 80). Unfortunately, no effective vaccine for gonococcal infection is available, due in part to the high variability of its surface antigens (8, 58, 65, 79), and treatment has depended heavily on antibiotics. This too is a problem in that gonococci show an ability to very rapidly develop resistance to antibiotics (4, 22, 63, 72).

The initial step of gonococcal infection is colonization of mucosal tissues, a complex process involving multiple components of both the bacteria and host cells. This process is critical for the production of an infection. If gonococci are unable to adhere to mucosal epithelial cells, they will be washed away with the normal flow of vaginal fluid (in women) or urine (in men). The primary adhesins on the gonococcal cell surface are the type IV pili, without which gonococci do not cause infection in humans (66, 67). Binding of gonococcal pili to host cells results in the induction of signal transduction pathways in the eukaryotic cell, which affects multiple cellular processes (3, 30, 33, 40, 48, 49, 52), and adherence of piliated gonococci results in a variety of rearrangements in the components of the cellular cytoskeleton (reviewed in reference 44). Little is known about the response of *N. gonorrhoeae* to this interaction, although our previous studies began to address this issue (16). In this work, we further these studies of the examination of gonococcal gene expression in response to contact with epithelial cells to test the hypothesis that attachment to epithelial cells signals the bacterium to modulate the expression of genes necessary for proliferation in the host.

Our previous studies showed that adherence to epithelial cells in culture results in changes of gene expression in gonococci and that this response is in part mediated by RpoH, a heat shock sigma factor (16). RpoH is a general transcriptional regulator in several bacteria, regulating a broad range of genes responding to different stimuli inside or outside the bacterium (1, 5, 46, 74, 77). Our previous work showed that RpoH played a role in virulence in *N. gonorrhoeae* in that depletion of RpoH reduced invasion of, but not adherence to, epithelial cells in culture. Here we show that some of the genes whose expression is induced in adherent gonococci require RpoH for this induction, while others are induced independently of RpoH. In addition, analysis of insertion mutants shows that one of the RpoH-dependent genes is important for invasion of but not adherence to epithelial cells, consistent with the previous conclusion that RpoH is important for the induction upon adherence of one or more genes that are important for subsequent steps in gonococcal infection. In addition, we show that one of the RpoH-independent genes is important for both adherence to and invasion of epithelial cells, suggesting the existence of

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multiple regulators in *N. gonorrhoeae* that mediate the response to host cell contact.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Escherichia coli* strain DH5α was used for all recombinant DNA manipulations and was grown in Luria broth supplemented as necessary with chloramphenicol (Cm) at 30 mg/liter, kanamycin (Kn) at 50 mg/liter, or erythromycin (Em) at 300 mg/liter. *Neisseria gonorrhoeae* strain MS11 (piliated [P^+], transparent [Tr] [57]) was grown in GC medium (Difco Laboratories, Sparks, MD) with Kellogg's supplements (35) in a humidified 5% CO_2 atmosphere. Em (3 mg/liter) and/or Cm (7 mg/liter) was added when necessary.

DNA manipulations. PCR was performed in a GeneAmp 9700 thermocycler (Applied Biosystems, Foster City, CA) using *Taq* DNA polymerase as described previously (16, 18). PCR products were purified using the QIAEX II gel extraction kit (QIAGEN, Valencia, CA) when necessary. Oligonucleotide primers were synthesized at the Michigan State University Research Technology Support Facility (MSU RTSF). DNA sequencing was done by the MSU RTSF. Restriction enzymes and T4 DNA ligase were from New England Biolabs (Beverly, MA) and were used as recommended by the manufacturer. Plasmid pKH35 (26) was used for molecular complementation constructions. *N. gonorrhoeae* transformation and genomic DNA isolation were performed as described previously (57, 60).

Construction of mutants. Plasmids containing genes of interest were from the *N. gonorrhoeae* clone set (7) and were used as targets for transposon mutagenesis. In vitro transposition was carried out using EZ::TN transposase (Epicenter Technologies, Madison, WI) and the modified transposon Tn*ErmUP* (15). The positions of transposon insertions were determined by PCR using primers homologous to the ends of the transposon (SqFP and SqRP; Epicenter) and to the ends of the gene in question and were corroborated by restriction analysis.

Infection assays. Human epithelial cell line A431 (ATCC CRL 1555) was grown at 37°C with 5% CO_2 in Dulbecco's modified Eagle medium (Gibco/Invitrogen, Carlsbad, CA) supplemented with 5% fetal calf serum (Gibco). Adhesion and invasion assays were performed as described previously (16). Assays were performed three or more times using fresh bacterial isolates, with each sample assayed in triplicate in each experiment. Adhesion frequencies were scored as the number of cell-associated CFU divided by the number of total CFU per well in that experiment. Invasion frequencies were calculated as the number of gentamicin-resistant (Gm^r) CFU per cell-associated CFU at the end of the experiment. Statistical analysis of data was done using the Student's *t* test, with *P* values indicated where appropriate.

RNA isolation and cDNA synthesis. RNA was isolated using TRIzol reagent (Invitrogen). An additional DNase treatment was performed using a Turbo DNA-free kit (Ambion, Austin, TX) to remove any remaining contaminating DNA. Purified RNA was eluted in RNase-free H_2O and stored at −80°C until further use. RNA was quantified spectrophotometrically, and quality was assessed by agarose gel electrophoresis. cDNA was synthesized by reverse transcription using Superscript II RNase H^- reverse transcriptase (RT; Invitrogen) using random hexamers (Invitrogen). Briefly, RNA was mixed with random hexamers and denatured at 75°C for 5 min. Mixtures were cooled to room temperature, and reaction buffer, deoxynucleoside triphosphates, and reverse transcriptase were then added to a total volume of 20 μl. First-strand synthesis was performed at 42°C for 50 min, and the reaction was terminated by heating to 75°C for 15 min. Controls without RT were included for all samples.

Microarray hybridization and data analysis. Global gene expression was measured using *N. gonorrhoeae* DNA microarrays composed of PCR amplicons of 2,035 of the 2,250 predicted open reading frames (ORFs) of the *N. gonorrhoeae* genome spotted onto glass slides and have been described previously (7, 16). Ten micrograms of total bacterial RNA was reverse transcribed and labeled using the CyScribe postlabeling kit (Amersham Biosciences, Piscataway, NJ). Cy3- and Cy5-labeled cDNAs were purified separately on QIAquick Cleanup columns (QIAGEN) and combined and concentrated to 15 μl using Microcon YM-30 microcentrifugation units (Millipore, Billerica, MA). Labeled probes were mixed with 1 μl of 1-mg/ml sheared, sonicated herring sperm DNA and denatured by boiling for 5 min followed by chilling on ice. DNA array slides were prehybridized, hybridized with labeled probes at 42°C overnight, and washed as described previously (16). Slides were scanned using a GenePix 4000B scanner (Axon Instruments, Union City, CA), and images were processed and analyzed using GenePix version 4.1 software. Four independent experiments (including a dye swap) were performed, and data analysis was performed as described previously (9, 16).

Quantitative RT-PCR. Oligonucleotides for genes of interest (*rpoH*, *groES*, NGO0372, NGO0376, and NGO1684) were designed using Primer Express software (Applied Biosystems), and sequences will be provided on request. Oligonucleotide primers for 16S rRNA were described previously (19, 55). Real-time PCR was performed using the SYBR green detection method (Applied Biosystems). Amplification reactions were carried out in a total volume of 15 μl and contained 1.5 μl 10× SYBR green PCR buffer, 1.5 μl 25 mM $MgCl_2$, 1.2 μl 10 mM deoxynucleoside triphosphate, 20 nM of each oligonucleotide, 0.15 μl AmpErase uracil-*N*-glycosylase (1 U/μl), 0.075 μl AmpliTaq Gold DNA polymerase (5 U/μl), and appropriately diluted template. The reaction mixtures were held at 50°C for 2 min, heated to 95°C for 10 min, and then cycled 40 times using parameters of 95°C for 15 s and 60°C for 30 s. Reactions were run on an ABI Prism model 7900HT sequence detection system (Applied Biosystems) at the MSU RTSF.

Three independent experiments were performed using freshly extracted RNA for each experiment (biological replicates). In each single experiment, dilutions of each reverse transcription product were loaded in triplicate for each pair of oligonucleotides. Data generated from real-time PCR experiments were processed and analyzed using Microsoft Excel. Differential gene expression was calculated using the relative expression method as recommended by Applied Biosystems. The relative amount of target was normalized to the internal control 16S rRNA (55), a house-keeping gene not regulated by RpoH or host cell contact (16). Data were subjected to statistical analysis using the Student's *t* test, and *P* values are indicated where appropriate.

RESULTS

Transcriptional analysis of gonococcal genes in an *rpoH* conditional strain. In our previous work we identified several genes, including *rpoH* and two genes known to be regulated by RpoH in *N. gonorrhoeae* (*groEL* and *groES*) (69) as being induced upon contact with epithelial cells (16). Based on these observations, we hypothesized that RpoH would mediate the induction of expression of some of these genes upon adherence to epithelial cells. To identify genes in addition to *groEL* and *groES* that are regulated by RpoH, global gene expression was examined in a gonococcal strain conditionally expressing *rpoH* (MPD288 [*rpoH*::Tn*EmUP lacIOP-rpoH* Cm]) (16). In this strain, the wild-type copy of *rpoH* on the chromosome was disrupted by insertion of the transposon Tn*EmUP* (15), and an additional copy of *rpoH* under control of a *lac* promoter was placed between *aspC* and *lctP* on the chromosome (26). MPD288 was grown in GC broth either with or without 20 μM isopropyl-β-D-thiogalactopyranoside (IPTG) for 6 h, and RNA was isolated from each culture. The RNA was then labeled and used to hybridize to *N. gonorrhoeae* DNA microarrays as described in Materials and Methods. Four independent experiments (including one dye swap) were performed, and data were analyzed as described previously (16).

Table 1 lists the average ratios of expression of genes identified previously as being induced upon host cell contact (16). As expected, the expression of *rpoH* was highly induced in the presence of IPTG (5.33-fold), consistent with the fact that *rpoH* is under control of a *lac* promoter in this strain. Also as expected, expression of *groES* and *groEL* were significantly increased (2.58-fold and 2.06-fold, respectively) in IPTG-treated samples. In addition, expression of NGO0376, predicted to encode a homolog of peptidyl-prolyl *cis-trans* isomerase B (28), was induced 2.71-fold in the presence of IPTG, indicating that this gene is regulated by RpoH. The expression ratios for the remainder of the genes induced upon host cell contact (16) were not significantly larger than twofold in the presence of IPTG, suggesting that they are not regulated by RpoH, at least not under the conditions tested.

Quantitative PCR analysis of selected host cell contact-induced genes in a conditional *rpoH* strain. To corroborate the

TABLE 1. Expression ratios of host cell contact-induced genes in the presence or absence of RpoH in vitro

ORF ID	Gene	Gene product	Expression ratio		SD[c]
			+Cells/−cells[a]	+IPTG/−IPTG[b]	
NGO0288	*rpoH*	RpoH, σ^{32}	2.61	5.33	2.56
NGO2094	*groES*	GroES, heat shock chaperone	2.39	2.58	0.47
NGO2095	*groEL*	GroEL, heat shock chaperone	2.10	2.06	0.17
NGO0376	*ppiB*	Peptidyl-prolyl *cis-trans* isomerase B	1.87	2.71	0.60
NGO1684		Conserved hypothetical protein	3.27	0.80	0.13
NGO1363	*mtrE*	MtrE, multidrug efflux pump	2.48	0.95	0.06
NGO1989		*Neisseria*-specific protein, uncharacterized	2.38	0.71	0.11
NGO0340	*cysK*	Cysteine synthase	2.34	1.06	0.36
NGO0238		Conserved hypothetical protein	2.34	1.01	0.33
NGO1210		Conserved hypothetical protein	2.24	NA[d]	
NGO0634		Hypothetical protein	2.03	1.16	0.51
NGO0372		ABC transporter	1.97	1.06	0.48
NGO0791		Conserved hypothetical protein	1.93	NA	
NGO1927		*Neisseria*-specific protein, uncharacterized	1.91	1.04	0.51
NGO0635		Hypothetical protein	1.91	1.19	0.35
NGO1083		Hypothetical protein	1.89	1.04	0.44
NGO0633	*nifU*	Fe-S scaffold protein	1.89	NA	
NGO1988		Conserved hypothetical protein	1.88	0.68	0.21
NGO0234	*hemN*	Porphyrin oxidoreductase	1.88	1.03	0.09
NGO0721		*Neisseria*-specific protein	1.85	1.49	0.76
NGO1366	*mtrR*	MtrR, multidrug efflux regulator	1.84	0.99	0.16
NGO1291		Conserved hypothetical protein	1.83	1.01	0.21
NGO0199	*rho*	Transcription termination factor Rho	1.79	0.70	0.17
NGO1515	*tyrC*	Prephenate dehydrogenase TyrC	1.76	NA	
NGO1493	*rpsT*	30S ribosomal protein S20	1.73	0.96	0.43
NGO1439	*aqpZ*	ATP-binding ABC transporter	1.72	0.87	0.14
NGO0989		Conserved hypothetical protein	1.69	NA	
NGO1831	*rpmC*	50S ribosomal protein L29	1.68	NA	
NGO1841	*rspJ*	30S ribosomal protein S10	1.68	1.08	0.15
NGO1668	*pgi*	Glucose-6-phosphate isomerase (Gpi)	1.67	1.01	0.56
NGO0637		Conserved hypothetical protein	1.67	0.98	0.41
NGO1773	*sdaA*	L-Serine dehydratase; L-serine deaminase	1.65	1.20	0.50
NGO2180		Conserved hypothetical protein	1.59	0.82	0.26
NGO0962	*pncB*	Nicotinate phosphoribosyltransferase	1.59	1.10	0.15
NGO5041	*ydhB*	Hypothetical protein, MS11 island	1.46	1.28	0.53
NGO1587	*mafB2*	Adhesin MafB2	1.41	1.01	0.23
NGO0276	*comA*	Competence protein (ComA)	1.32	0.96	0.26

[a] Gene list and ratios are from reference 16. +Cells, gene expression in gonococci adherent to A431 cells; −cells, gene expression in gonococci grown in the absence of cells.
[b] +IPTG, growth in GC broth plus 20 μM IPTG; −IPTG, growth in GC broth alone.
[c] SD, standard deviation.
[d] NA, not available.

microarray results and confirm that expression of NGO0376, but not NGO0372 or NGO1684, is induced by RpoH, real-time RT-PCR was performed. *N. gonorrhoeae* strain MPD288 was grown with and without the inducer IPTG as for the microarray experiment described above. RNA was isolated, and cDNA was synthesized using random primers as described in Materials and Methods. Gene-specific primers for NGO0372, NGO 0376, and NGO1684, as well as for *groES* and *rpoH* (as controls), were designed and used in real-time PCR to determine the relative levels of transcription of these genes in IPTG-treated and untreated samples. The relative amounts of products were normalized to an internal 16S rRNA control, as it is a housekeeping gene whose expression is not influenced by RpoH (16).

As expected, the expression of *rpoH* was highly induced in bacteria grown in the presence of IPTG (7.5-fold), consistent with the fact that *rpoH* is under control of a *lac* promoter in this strain. NGO1684, annotated as encoding a conserved hypothetical protein (6), is the gene whose expression was most highly induced in gonococci adhering to A431 cells (3.27-fold [Table 1]) (16). Microarray analysis of the conditional *rpoH* strain MPD288 showed that this gene was not more highly expressed in inducing conditions, suggesting that it is not regulated by RpoH (ratio, 0.80 ± 0.13 [Table 1]). Consistent with this observation, real-time PCR analyses show that NGO1684 is not induced in MPD288 grown with IPTG and may actually be slightly repressed under these conditions (ratio, 0.43 ± 0.12; $P < 0.0001$ [Fig. 1]). NGO0372, annotated as encoding a periplasmic amino acid binding component of an ABC transporter (6), was also shown to be significantly up-regulated in adherent gonococci (1.97-fold [Table 1]) (16). Like NGO1684, NGO 0372 was not significantly induced by IPTG in the DNA microarray analysis of the conditional *rpoH* strain, MPD288 (ratio 1.06 ± 0.48 [Table 1]) nor was it significantly differentially expressed in the real-time PCR analyses (ratio, 0.99 ± 0.40; $P = 0.958$ [Fig. 1]). In contrast, *groES*, known to be regulated by RpoH in several bacterial species, including *N. gonorrhoeae* (16, 69), was induced more than threefold in IPTG-treated

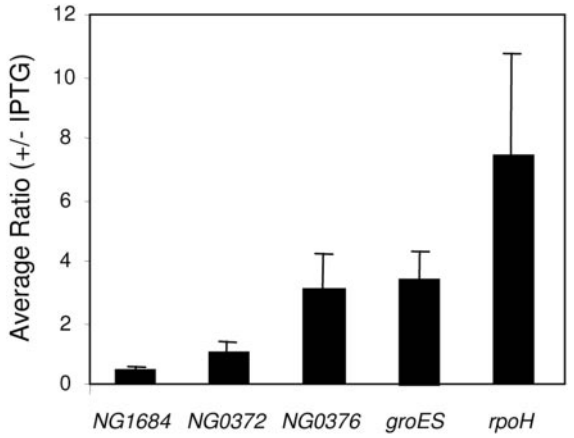

FIG. 1. Real-time quantitative PCR analysis of expression of NGO 1684, NGO0372, NGO0376, *groES*, and *rpoH* in the conditional *rpoH* strain MPD288 grown with and without inducer (IPTG). The expression of each gene was normalized to that of the housekeeping gene 16S rRNA. Ratios were calculated as the expression level of a gene in MPD288 grown with 20 μM IPTG divided by that from MPD288 grown without IPTG. Data presented are averages from three independent experiments, and error bars represent standard deviation.

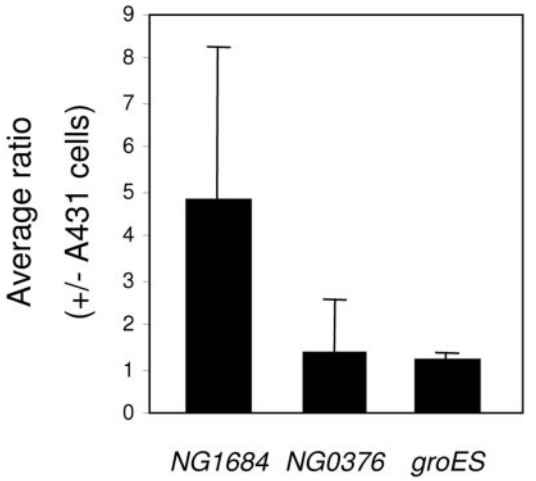

FIG. 2. Real-time quantitative PCR analysis of expression of NGO 1684, NGO0376, and *groES* in MPD288 depleted for RpoH and adherent to A431 cells. RNA isolated from MPD288 depleted for RpoH adherent to A431 cells (+) or grown in the absence of A431 cells (−) was reverse transcribed using random primers. Real-time PCR was performed with primers specific for NGO1684, NGO0376, *groES*, and 16S rRNA as described in the text. Ratios were calculated as the expression level of a gene in adherent MPD288 divided by that of MPD288 grown in cell-free culture. Data presented are averages from three independent experiments, and error bars represent standard deviation.

samples as determined by real-time PCR (ratio, 3.39 ± 0.92; $P = 0.0002$ [Fig. 1]). DNA microarray analyses showed expression of NGO0376 to be induced nearly threefold in IPTG-induced samples, and real-time PCR results are consistent with this observation in that an expression ratio of 3.12 ± 1.09 was determined ($P = 0.0004$) (Fig. 1). These results indicate that while NGO0376 and *groES* are regulated by RpoH, NGO1684 and NGO0372 are not.

Induction of NGO0376 and *groESL* upon host cell contact is mediated by RpoH. Previous results showed that NGO0376 was induced in gonococci adherent to epithelial cells (16). Results obtained by both microarray analysis (Table 1) and real-time PCR (Fig. 1) showed that NGO0376 was regulated by RpoH in standard laboratory medium (GC broth). Thus, we next asked whether the induction of NGO0376 expression in adherent gonococci was dependent on RpoH. *N. gonorrhoeae* strain MPD288, which conditionally expresses *rpoH*, was grown in the absence of the inducer, IPTG, to deplete RpoH in the cell, and then used to infect A431 cells. For comparison, RpoH-depleted bacteria were grown in cell culture medium without cells. At 3 h postinfection (p.i.), cells were washed to remove nonadherent bacteria, and bacteria adherent to cells were lifted by lysing the epithelial cells with saponin. Adherent bacteria grown in the absence of cells were harvested by centrifugation, and RNA was isolated as described in Materials and Methods. cDNA was made from the RNA using random primers, and levels of expression of NGO0376, *groES*, and NGO1684 in the samples were quantified by real-time PCR. NGO1684 and *groES* were used as negative and positive controls, respectively. NGO1684, while induced in gonococci adherent to epithelial cells (16), did not appear to be regulated by RpoH in either microarray (Table 1) or quantitative PCR experiments (Fig. 1). *groES* expression has been shown to be regulated by RpoH in *N. gonorrhoeae* (Table 1; Fig. 1) (16, 69) as well as induced upon host cell contact (16).

As expected, the expression of *groES* in adherent gonococci depleted of RpoH was similar to the levels observed in gonococci grown in cell culture medium alone, indicating that *groES* was no longer induced by contact with host cells in the absence of RpoH (ratio, 1.22 ± 0.17; $P = 0.040$ [Fig. 2]). A similar expression pattern was observed for NGO0376, which was also no longer induced in adherent gonococci in the absence of RpoH (ratio, 1.39 ± 1.20; $P = 0.357$ [Fig. 2]). Interestingly, expression of NGO1684 was still induced in adherent gonococci depleted of RpoH (ratio, 4.84 ± 3.47; $P = 0.042$ [Fig. 2]), suggesting that another regulatory mechanism, independent of RpoH, is involved in modulating the expression of this gene in response to contact with epithelial cells. Taken together, these results show that induction upon host cell contact of NGO0376 and *groES*, but not NGO1684, is mediated by RpoH.

Identification of a putative RpoH-like promoter upstream of NGO0376. Several genes of *E. coli* have been identified as being transcribed from σ^{32}(RpoH)-containing RNA polymerase, leading to the determination of a consensus RpoH-regulated promoter (37, 41, 76). Tauschek and coworkers identified a putative RpoH-binding site upstream of *groES* in *N. gonorrhoeae*, which is near a transcriptional start site that was more active following 10 min of pulsing at 42°C (69). This promoter sequence shares high similarity (13/20 nucleotides match) to the consensus RpoH promoter identified in *E. coli* (76). Analysis of the promoter region of NGO0376 revealed a sequence with high similarity to both the gonococcal *groES* promoter and the consensus *E. coli* RpoH promoter (13/20 nucleotides match). The identification of a putative RpoH-binding site upstream of NGO0376 is consistent with the observation that this gene is regulated by RpoH and suggests that the regulation is direct, with NGO0376 being transcribed from a σ^{32} (RpoH)-containing RNA polymerase in *N. gonorrhoeae*.

Construction of gonococcal strains with mutations in host cell contact-induced genes. Since our previous results indicated that several genes were induced in gonococci adhering to epithelial cells (16), we hypothesized that one or more of those genes would be important for subsequent steps of a gonococcal infection. To test this, several host cell contact-induced genes were selected for further investigation of their roles in gonococcal infection. The rationale for the selection of genes to mutate was as follows: NGO0340, NGO0634, NGO1363, NGO1684, NGO1989, and NGO2094 were selected based on the observation that they showed a ≥2-fold increase in expression in adherent gonococci (16) (Table 1). While NGO0238 and NGO1210 were also induced ≥2-fold upon adherence, neither was present in the clone set used as targets for mutagenesis (7), and therefore we were not able to mutagenize them. NGO2095 *(groEL)* is the second gene of the *groES-groEL* operon (69), and only NGO2094 *(groES)* was selected for mutational analysis. NGO0372 was selected as it was induced nearly twofold upon adherence and was also found to be differentially expressed in other experiments (J. Lenz and C. G. Arvidson, unpublished results) and was therefore of interest. NGO0376 was selected since it was found to be dependent on RpoH for induction in adherent gonococci (Table 1; Fig. 1 and 2), and NGO1366 *(mtrR)* was selected as it encodes a previously characterized transcriptional regulator (25).

Plasmids containing genes of interest were insertionally inactivated by in vitro transposition using the transposon Tn*ErmUP* as described previously (15, 16). Plasmids containing mutated genes were then used to transform *N. gonorrhoeae* strain MS11 to Em^r. Allelic exchange between the wild-type copy on the chromosome and transforming DNA containing the mutant copy with the Em^r determinant should result in null mutations of each of these genes. Not surprisingly, despite several transformation attempts, we were unable to construct a gonococcal *groES* null mutant, as evidenced by the fact that no Em^r transformants were obtained. Since *groES* is essential in other bacterial species (20), it is likely that it is essential in gonococci as well, although additional experiments will be required to prove this. We were also unable to construct null mutations in three additional genes, NGO0372, NGO0634, and NGO1363. The reasons for this were not clear, but for this work these genes were not studied further. Once mutants were successfully constructed in *N. gonorrhoeae* strain MS11, each mutant was backcrossed with the parent strain by transformation with genomic DNA. This was to ensure that the mutation was caused by the insertion of the transposon in the gene of interest. A list of the mutant strains constructed and the gene mutated in each is shown in Table 2.

TABLE 2. Mutants constructed in host cell contact-induced genes

Gene	ORF ID	Mutant	RpoH dependent[a]
cysK	NGO0340	M0340	No
	NGO0372	ND[b]	No
ppiB	NGO0376	M0376	Yes
	NGO0634	ND	No
mtrE	NGO1363	ND	No
mtrR	NGO1366	M1366	No
	NGO1684	M1684	No
	NGO1989	M1989	No
groES	NGO2094	ND	Yes

[a] Regulation is mediated by RpoH as determined by DNA microarray analysis (Table 1) and quantitative PCR (Fig. 1).
[b] ND, no mutant obtained.

Analysis of mutants for interactions with epithelial cells in culture. Once gonococcal strains were constructed with insertion mutations in individual genes identified as being induced upon contact with epithelial cells, we next asked whether any of the mutations affected the ability of gonococci to adhere to and/or invade A431 cells in culture. Wild-type MS11 and mutant strains were grown overnight on GC agar and then used to infect A431 cells at a multiplicity of infection of 10 as described in Materials and Methods.

Figure 3A summarizes the results of three independent experiments in which adherence was measured at 3 h p.i. and adhesion frequencies were expressed as numbers of percent cell-associated CFU as a function of the total number of CFU in the well at the end of the experiment. These results show that insertion mutations in NGO0376, NGO1366, NGO1684, and NGO1989 had no appreciable affect on gonococcal adherence to A431 cells compared to the wild-type parent, MS11. However, adherence of mutant M0340 was significantly reduced, with an adhesion frequency more than threefold lower than that determined for MS11 ($32.9 \pm 9.3\%$; $P < 0.0001$). A possible explanation for this phenotype could be that the mutant expressed a variant *pilE* gene, which encodes pilin, the major subunit of the gonococcal pilus (45). Type IV pili are the primary adhesins for gonococci (66) and undergo antigenic variation at a high frequency (59); thus, a variant pilin could alter the ability of the strain to adhere to cells in culture. To test this possibility, the *pilE* genes from M0340 and from the wild-type parent strain, MS11, were PCR amplified, and DNA sequences were determined. The sequencing results showed that the two *pilE* sequences were identical at the nucleotide level, indicating that the reduced adhesion phenotype of M0340 is not due to production of a variant pilin in the M0340 mutant strain.

Figure 3B summarizes the invasion assay results obtained from three independent assays performed in triplicate. Assays were performed as described previously (16), and invasion was scored as the number of Gm^r CFU per cell-associated CFU at 7 h p.i. The results of these experiments show that two mutants, M0340 and M0376, were significantly reduced in their ability to invade A431 cells at 7 h p.i. Mutant M0340, which showed reduced adherence to A431 cells (Fig. 3A), also invaded cells ~70% ($70.1\% \pm 10.4\%$; $P < 0.0001$) as well as the wild-type parent strain, indicating that this mutant is generally deficient in its ability to interact with epithelial cells. A second mutant, M0376, was also reduced in its ability to invade A431 cells, invading ~40% ($40.9\% \pm 8.5$; $P < 0.0001$) as well as the wild-type MS11, although it appeared to adhere to cells as well as MS11 (Fig. 3A). The other three mutants assayed— M1366, M1684, and M1989—invaded with essentially the same frequencies as the wild-type strain.

Molecular complementation of the NGO0340 and NGO0376 mutations. In order to demonstrate that the reduced adhesion and invasion phenotypes observed for strains M0340 and M0376 were indeed due to insertion mutations in NGO0340 and NGO0376, molecular complementation experiments were performed in which each of the genes was cloned and expressed in *trans* in the corresponding insertion mutant

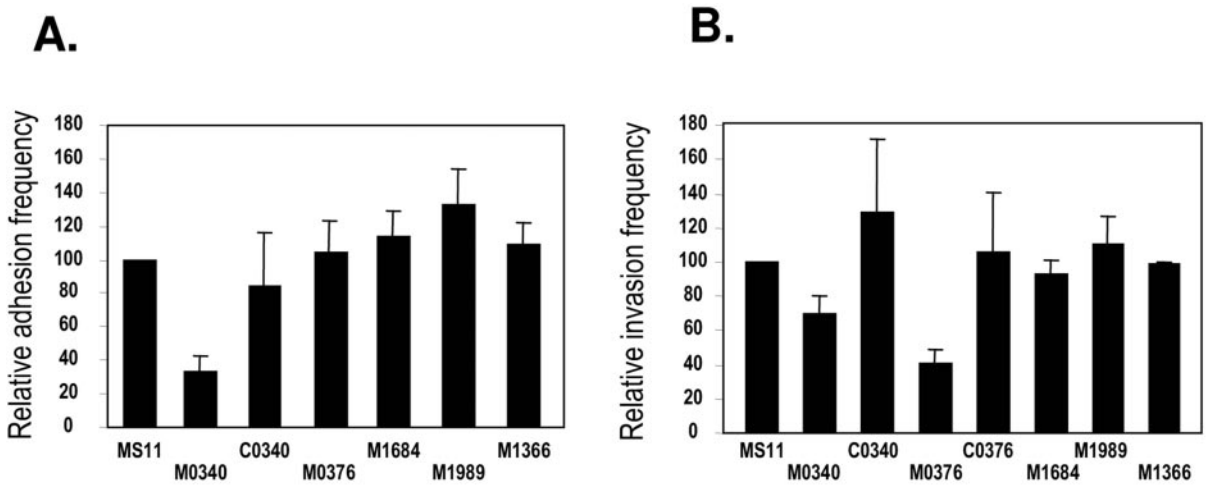

FIG. 3. Interaction of *N. gonorrhoeae* mutants with A431 cells. (A) Adherence to A431 cells. Adhesion data are presented as relative adhesion frequency, normalized to the wild-type control (MS11), which was arbitrarily set at 100%. (B) Invasion of A431 cells. Invasion data are presented as relative invasion frequency, normalized to MS11, which was arbitrarily set at 100%. These data are averages from three independent experiments performed in triplicate, and error bars indicate standard deviation.

strains. NGO0340 and NGO0376, along with 400 to 500 bp of 5′- and 3′-flanking sequences, were PCR amplified and cloned into a derivative of the plasmid pKH35 in which the P_{lac}-*lacI*^{q} fragment had been removed, to generate pC340 and pC376. Adjacent to the cloned genes on these plasmids is a marker for Cm^r, and flanking the cloned gene marker construct are two genes, *aspC* and *lctP* of the *N. gonorrhoeae* chromosome, in between which it has been reported that large sequences can be inserted without being deleterious to the cell (16, 26). Transformation of *N. gonorrhoeae* with pC340 and pC376 and selection for Cm^r resulted in recombination between the incoming DNA and the *aspC-lctP* region of the chromosome. The resulting heterodiploids are such that the chromosomal copy of NGO0340 or NGO0376 had the Tn*ErmUP* insertion, and an additional wild-type copy of the corresponding gene is at the *aspC-lctP* locus. Transformants were confirmed as having both a wild-type and transposon-mutated copy of NGO0340 or NGO0376 by PCR using combinations of gene-specific and transposon-specific primers.

The resulting strains, C340 and C376, were then used to infect A431 cells to measure adherence and invasion as described in Materials and Methods, and the data are shown graphically in Fig. 3. The complemented mutant, C340, adhered to and invaded A431 cells as efficiently as the original wild-type parent strain, MS11 (P values of 0.436 and 0.296, respectively). The complemented mutant, C376, also invaded A431 cells as efficiently as the original wild-type parent strain ($P = 0.798$). These results indicate that providing the wild-type NGO0340 and NGO0376 in *trans* indeed complemented the mutations, restoring the adherence and invasion capabilities of the two mutant strains.

DISCUSSION

Adherence to mucosal epithelial cells is a critical first step in gonococcal infection. As gonorrhea is a sexually transmitted disease, transmission to a new host often results in the bacterium entering an environment very different from the one from which it came, and the initial interaction with epithelial cells has the potential to serve as a signal to the bacterium that it is in a new host. Previous studies from our laboratory have demonstrated that attachment of gonococci to epithelial cells results in the modulation of expression of several genes (16). Genes differentially expressed in adherent gonococci included those involved in a variety of functions, such as glycolysis, amino acid metabolism, protein synthesis, protein transport, and regulation of gene expression. In particular, we found that *rpoH*, which encodes the heat shock sigma factor, σ^{32} (RpoH), was induced in adherent gonococci and that this induction was necessary for optimal invasion of epithelial cells.

RpoH is a critical regulator of the bacterial heat shock response and is highly conserved in bacteria (47, 81). Studies in other bacteria have shown that RpoH is a general transcription factor involved not only in the heat shock response but also in the response to osmotic pressure, misfolded proteins, nutrient limitation, and other stimuli (1, 2, 5, 78). Our observation that RpoH is involved in modulating gene expression in gonococci upon contact with host cells suggests that this interaction is yet another stress response mediated by RpoH in a bacterium.

The goals of this work were twofold. First, we sought to determine which genes induced upon adherence to epithelial cells require RpoH for this induction. Second, we examined several host cell contact-induced genes to determine which, if any, play a role in adherence to and invasion of epithelial cells.

NGO0376, encoding a putative rotamase, was found to be dependent on RpoH for induction upon contact with epithelial cells. Three pieces of evidence support this conclusion. First, in a standard laboratory medium (GCB), expression of this gene was significantly induced in the conditional *rpoH* strain grown in the presence of inducer as determined by DNA microarray analysis (Table 1) and quantitative RT-PCR (Fig. 1), indicating regulation by RpoH. Second, when the conditional *rpoH* strain, MPD288, was grown in RpoH depletion conditions (no IPTG),

induction of NGO0376 expression in adherent gonococci was abated (Fig. 2), indicating that RpoH was required for cell contact-mediated induction. Third, a putative RpoH-binding site with significant sequence similarity to RpoH-dependent promoters was identified upstream of NGO0376 (data not shown). Similar observations were made for *groES*, a well-characterized RpoH-dependent gene in several bacteria including *N. gonorrhoeae* (16, 23, 38, 69, 75). Taken together, these results demonstrate that like *groEL* and *groES*, NGO 0376 is part of the gonococcal RpoH regulon and requires RpoH for induction upon contact with epithelial cells.

In contrast to our observations that *groEL* and *groES* and NGO0376 are induced in adherent gonococci in an RpoH-dependent manner, NGO1684, whose expression was the most highly induced in gonococci adherent to A431 cells (16), appeared to be RpoH independent. Expression of NGO1684 in the conditional *rpoH* strain in the presence of inducer was essentially the same as in its absence (Table 1). However, when the conditional *rpoH* strain, MPD288, was grown in RpoH depletion conditions and used to infect A431 cells, expression of NGO1684 in adherent gonococci was still induced (Fig. 2). These results clearly indicate that the induction of NGO1684 in adherent gonococci is independent of RpoH and is likely mediated by another as-yet-unidentified regulator.

We previously showed that several genes were induced in gonococci adhering to epithelial cells (16). This led to the hypothesis that one or more of those genes would be important for adherence to and/or invasion of epithelial cells. To test this, mutations in several host cell contact-induced genes were constructed and analyzed for their effects on adherence to and invasion of A431 cells in culture.

We were successful in isolating null mutants of NGO0340, NGO0376, NGO1366, NGO1684, and NGO1989 (listed in Table 2) but were unable to isolate transformants with insertion mutations in *groES*, NGO0634, NGO0372, and NGO1363, in spite of several attempts. It is possible that these genes are essential in *N. gonorrhoeae* and is very likely for *groES*, which is known to be essential in other bacterial species (20). However, there have been reports of *mtrE* (NGO1363) mutants isolated in other *N. gonorrhoeae* strains (14); thus, there may be other explanations for our inability to construct a *mtrE* mutant in MS11. NGO0372 encodes a putative ABC transporter component (6) and is induced in adherent gonococci in an RpoH-independent manner. Whether or not the putative ABC transport system is essential will require further experimentation. Another explanation for our inability to obtain some of these mutants is that there may be polar effects on genes downstream of those we attempted to mutate. Additional experiments, such as using plasmid constructs with more flanking sequences on either side of the transposon insertion to facilitate homologous crossover and/or the development and use of a nonpolar transposon might result in viable mutants of these genes. To prove that either of these is essential will require the construction of conditional mutants.

Assays for adherence to A431 epithelial cells using the six transposon insertion mutants isolated (listed in Table 2) showed that one mutant, M0340, was significantly reduced in its ability to adhere to cells (Fig. 3A). This mutant was also reduced in its ability to invade A431 cells (Fig. 3B). Since invasion is scored as a function of adherence (Gm^r CFU per cell-associated CFU), the observed reduction in invasion frequency indicates that each step (adherence and invasion) is defective in this mutant and that the protein encoded by this gene, thought to be involved in cysteine biosynthesis, is important for gonococcus-host cell interactions. While expression of NGO0340 is induced in adherent gonococci, this induction is independent of RpoH (Table 1; Fig. 1); therefore, our previous observation that depletion of RpoH in gonococci reduces its ability to invade A431 cells (16) is not due to a lack of induction of NGO0340 expression. However, this does provide evidence for the existence of additional regulators that are involved in the host cell contact response.

In the gonococcal genome database (6), NGO0340 is annotated as encoding an *O*-acetylserine(thiol)-lyase A, which catalyzes the last step in the synthesis of L-cysteine (EC 2.5.1.47). In *E. coli* and the closely related *Salmonella enterica* serovar Typhimurium, there are two genes encoding *O*-acetylserine-(thiol)-lyase A, *cysK* and *cysM* (10); however, only one (annotated as *cysK*) has been identified in the gonococcal genome. Cysteine has been shown to be very important for growth of gonococci in that cysteine is required for colony formation on chemically defined agar medium (11), and cystine (formed from cysteine in oxidizing conditions) is a limiting factor in continuous culture of gonococci (34). Cysteine is an essential amino acid, not synthesized by the human host; thus, it is likely that it will be limiting in the natural environment of the gonococcus. Cysteine, as one of two sulfur-containing amino acids, serves as a source of organic sulfur in the bacterial cell and is also critical for the folding of many proteins via the formation of disulfide bonds. It is this feature that may account for our observations that *cysK* is necessary for gonococcal host cell interactions. The interaction between gonococci and host cells involves several proteinaceous structures on the surface of the bacterium, including pili (66), opacity proteins (43, 71, 73), and porins (31, 36). Cysteine residues are highly conserved in the otherwise highly antigenically variable pilin, the major protein component of the gonococcal pilus (61), and are critical in the formation of disulfide bonds during assembly of the pilus fiber (50). As pili are critical for gonococcal attachment to epithelial cells, this could explain why *cysK* mutants do not adhere well to A431 cells (Fig. 3A), although since pili do not promote invasion and may even impede the process (43, 62), this does not explain our observation that M0340 invades A431 cells less efficiently than the wild type. The reason for this observation is less clear and will require additional experimentation.

A second mutant, M0376, adhered to A431 cells essentially the same as the wild type (Fig. 3A). However, this mutant did not invade A431 cells well at all, ~40% as efficiently as the wild type (Fig. 3B). Furthermore, the induction of expression of NGO0376 in adherent gonococci is dependent on RpoH (Table 1; Fig. 1 and 2), consistent with our prior observation that RpoH is necessary for optimal invasion of A431 cells (16). In the gonococcal genome database (6), NGO0376 is annotated as encoding a peptidyl-prolyl *cis-trans* isomerase B (also referred to as rotamase B, PPIase, and PpiB). Rotamases facilitate the folding of proteins by catalyzing the *cis-trans* isomerization of proline imidic peptide bonds of proline-containing peptides and were first identified as cyclophilins in higher organisms that were targets of the immunosuppressive drug cyclosporine (21, 68). Analysis of the many sequenced

genomes show that rotamases are present in nearly all prokaryotic and eukaryotic organisms, many having several rotamases with slightly differing functions and characteristics (reviewed in reference 24). Rotamases can be divided into three families: (i) those of the cyclophilin family, which are homologous to cyclosporine binding proteins; (ii) those of the FKBP (*FK*506 *b*inding *p*rotein) family, which bind another immunosuppressive drug, FK506, and are not similar in sequence to cyclophilins (27, 64); and (iii) those of a recently identified family called parvulins, so named for their small size (10.1 kDa) (53, 54). Bacterial rotamases are found in the cytoplasm and periplasm and can also be surface exposed (17, 28, 29).

Rotamases play a role in the proper folding of many bacterial proteins, including virulence factors in bacterial pathogens. A *Legionella pneumophila* rotamase mutant was shown to invade the amoeba *Acanthamoeba castellanii* 10-fold less well than the wild type (56). Hermans and coworkers recently identified a surface-exposed rotamase, SlrA, in *Streptococcus pneumoniae* that was important for colonization but not invasive disease in a mouse model of infection (29). In addition, *slrA* mutant pneumococci were impaired in their ability to adhere to multiple cell lines in culture, clearly demonstrating a role for SlrA in pneumococcus-host cell interactions. Of the nine rotamases identified in *E. coli*, five are cytoplasmic and four are periplasmic (17). A quadruple mutant in which each periplasmic rotamase gene was inactivated was viable but reduced in its ability to assemble pili (both type I and P) (32). As pili are adhesins in uropathogenic *E. coli* that mediate their attachment to cells in the urogenital tract, a defect in pilus assembly would likely affect the ability of uropathogenic *E. coli* to interact with host cells in an infection. Our observation that a null mutation in a gene encoding a putative rotamase in *N. gonorrhoeae* results in a defect in invasion is consistent with these observations that rotamases are important in pathogen-host interactions.

There are five putative rotamases encoded in the gonococcal genome (6): one of the parvulin family (NGO0766 or *ppiD*), two of the cyclophilin family (NGO0376 or *ppiB* and NGO0544 or *ppiA*), and two of the FKBP family (NGO0981 or *slyD* and NGO1225 or *mip*). Leuzzi and coworkers recently reported that NGO1225, which encodes an FKBP-type rotamase, is involved in persistence of gonococci in macrophages (42). Indeed, NGO1225 is annotated as encoding a macrophage infectivity potentiator (MIP), one of a family of proteins found on the surface of several intracellular pathogens that plays a role in the interactions between the pathogen and phagocytic cells. Null mutations in NGO1225 are viable, and the mutants appear not to be defective in production of pili or Opa proteins and are bound by and taken up by macrophages as well as the wild-type parent strain (42). However, NGO1225 mutants appeared to be more sensitive to killing by the macrophages, as fewer intracellular bacteria were recovered from macrophages infected with the mutant than the wild type. This phenotype was specific for macrophages, as the mutant behaved similarly to the wild type in adhesion, invasion, and intracellular survival assays using ME-180 (human cervical epithelial) cells. Little is known of the roles played by the other gonococcal rotamases.

NGO0376 is the only rotamase gene identified as being regulated upon contact with epithelial cells (16). DNA microarray experiments showed that expression of NGO0544, NGO0766, NGO0981, and NGO1225 was essentially the same in gonococci grown in the absence of epithelial cells and in gonococci adhering to epithelial cells. Regulation of NGO0376 upon contact with epithelial cells is dependent on RpoH (Table 1; Fig. 1 and 2), similar to regulation of expression of rotamase genes in *E. coli*, which varies for the many rotamases identified in this bacterium. Not surprisingly, most are controlled by stress regulators such as CpxR and the heat-shock sigma factors, σ^{32} (RpoH) and σ^{E} (RpoE) (12, 13, 51).

In this work, we showed that an NGO0376/*ppiB* mutation affects invasion of but not adherence to epithelial cells (Fig. 3). Our previous studies showed that *ppiB* expression is increased when gonococci are adherent to cells (16). These observations suggest that gonococci may be responding to an increase in the synthesis of proline-containing proteins that are important for the invasion process and need to increase the production of PpiB to facilitate proper folding of these proteins. That PpiB is cytoplasmic suggests that its target proteins are at least folded in the cytoplasm, if not remaining there. The identity of these targets remains a mystery.

In summary, we have shown that adherence of gonococci to epithelial cells, a critical early step in infection, results in the modulation of expression of several genes that are important for subsequent steps. In particular, two of these genes, NGO0340 and NGO0376, encoding cysteine synthetase and rotamase, respectively, are likely important in the proper synthesis and assembly of proteins that are needed following the attachment step. That the heat shock sigma factor, RpoH, in part mediates this regulation is consistent with observations in other bacteria that this regulatory system responds to environmental stresses, and it indicates that signaling is occurring across the bacterial membrane. Additionally, the identification of genes that are induced by host cell contact independent of RpoH indicates that there are multiple regulators involved in this response. Further characterization of this regulatory network will be key to a more in-depth understanding of the communication between gonococci and the human host.

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