Two-Component Systems in *Haemophilus influenzae*: a Regulatory Role for ArcA in Serum Resistance

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Knockout mutations were constructed in the *arcA* gene of a virulent type b strain of *Haemophilus influenzae*, and the behavior of the resulting mutants was investigated in a number of conditions that mimicked distinct steps in the natural infection pathway. In *arcA* mutants, synthesis of capsule and lipooligosaccharide (LOS) and growth in synthetic media were unaltered compared to synthesis of capsule and LOS and growth in synthetic media in the wild-type *H. influenzae* type b parent strain. However, the virulence of the *arcA* mutants for BALB/c mice was significantly reduced. Upon exposure to human blood or serum, the *arcA* mutants showed markedly reduced survival compared with the survival of its wild-type parent. Serum resistance could be fully restored by complementation in *cis* with the *H. influenzae arcA* gene but not by complementation in *cis* with the homologous gene from *Escherichia coli*. The proteomes of wild-type and mutant bacteria were markedly different, especially under anaerobic conditions, underscoring the global regulatory role of ArcAB in *H. influenzae*. Evaluation of antibody titers and classical complement activities in various serum samples pointed to complement-mediated bactericidal activity as the factor that distinguishes between the *arcA* mutant and wild-type phenotypes. Comparative analysis of the membrane fractions of the *arcA* mutants and the wild-type strain revealed several ArcA-regulated proteins, some of which may be implicated in the serum hypersensitivity phenotype.

The only known natural host of *Haemophilus influenzae* is humans. Carriage of unencapsulated *H. influenzae* in the nasopharyngeal area is common, especially among children, and is considered a probable source of infection in otitis media, sinusitis, and pneumonia (51). Life-threatening *H. influenzae* meningitis is caused mainly by encapsulated type b strains; this is attributed to several factors, including the resistance of these strains to bactericidal activities of blood. Passage from the upper respiratory mucosa via the general circulation to the meninges requires successive adaptations of a bacterium's physiology in order to cope with the environmental changes that it encounters. Although the roles of the type b capsule (61) and lipooligosaccharide (LOS) (57) in invasive disease have been clearly demonstrated, we know little about the roles of other virulence factors in *H. influenzae* infections, not in the least because of the lack of reliable animal models.

We hypothesized that the capacity of *H. influenzae* to swiftly adapt its physiology to match environmental conditions, such as changes in oxygen availability, is likely a virulence-associated trait. Two-component systems that are regulators of gene transcription in response to environmental signals have been implicated in virulence in a number of bacterial species, including *Bordetella pertussis, Salmonella enterica* serovar Typhimurium, and *Shigella flexneri* (5, 18, 53). No such role has yet

been demonstrated for the ArcAB system involved in oxygen-dependent regulation of gene expression, although oxygen levels affect the expression of several virulence genes in other human pathogens (2, 38, 40). In this study, *H. influenzae arcA* mutants were constructed and systematically analyzed with respect to cell wall constituents, in vitro growth rates, interactions with human cells, and protein expression profiles. The most significant difference that we were able to demonstrate between the wild-type and *arcA*-deficient strains was an increased sensitivity to human serum bactericidal activity in the mutants. We hypothesize that this aspect of the ArcA phenotype could be associated with virulence in humans.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *H. influenzae* type b strain ATCC 10211 was used as a source of PCR products and as a background for all gene replacement studies. Some cloning was done in *H. influenzae* strain KW20. *Escherichia coli* DH10B was used as a source for PCR of the *E. coli arcA* gene. *H. influenzae* strains were grown in complete BHI medium, which consisted of 3.7% brain heart infusion medium (Difco) supplemented with IsoVitaleX (Becton Dickenson), NAD (2 μg/ml), and hemin (10 μg/ml). Alternatively, strains were grown in MIc minimal medium (3). The final concentrations of antibiotics for *H. influenzae* markers were as follows: ampicillin, 10 μg/ml; tetracycline, 5 μg/ml; kanamycin, 7 μg/ml; and streptomycin, 50 μg/ml. Luria broth was used for growth of all *E. coli* strains. Michelle Gwinn kindly provided the *H. influenzae* KW20 *arcA*::*kan* mutant (26). Mutations were introduced into the virulent *H. influenzae* ATCC 10211 background by transformation with purified chromosomal DNA obtained from the KW20 recombinants.

In vivo virulence model. The virulence of *H. influenzae* strains was tested by using a mouse septicemia model. Inbred male BALB/c mice (Charles River) that weighed 18 to 22 g and were 6 weeks old were housed under standard temperature and relative humidity conditions with a 12-h light schedule. Food and water were available ad libitum. The bacterial inocula were prepared from overnight

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TABLE 1. Oligonucleotides used for PCR of the upstream and downstream fragments used in *arcA* knockout constructs[a]

Oligonucleotide	Sequence (5′-3′)
arcA-up1	GCTCTAGA**GGCCTCGACCCCC**ATCTTTAACGGGTAGTTAAACA
arcA-up2	GGGGTACC**GGCCATGCGGGCC**CTGTGCAACATTATTGTAACAA
arcA-down1	GCTCTAGA**GGCGCGCC**AGTGATTGTTCTACATAAAAATT
arcA-down2	GGGGTACC**GCGGCCGC**TGTATTCTTTACGTGATAGTAAT

[a] Restriction enzyme sites used for cloning are indicated by boldface type.

cultures on chocolate agar plates, which were grown at 37°C under 5% CO_2. The bacteria were resuspended to a density of 0.36 absorbance unit at 600 nm, corresponding to 7.2×10^7 CFU/ml, in a saline solution, and 10-fold dilutions were prepared. Each dilution was verified by colony counting and was injected intraperitoneally (0.5 ml per mouse) as a 1:1 mixture with enhancement medium (2% mucin and 2% bovine hemoglobin) (7). Groups of five mice were inoculated with each bacterial dose. The animals were observed for 4 days after inoculation. A median lethal dose was calculated by Probit analysis (16). The animal experiments were performed in full compliance with Italian national legislation and with the Glaxo Wellcome company policy on the care and use of animals.

Plasmid construction. A suicide plasmid for *H. influenzae* was constructed by insertion of a PCR fragment containing *gfp-tet* (the green fluorescent protein coding sequence and the *tetR* and *tetA* genes from Tn*10*) into the vector pMTL22 (9) between the *Bam*HI and *Nsi*I sites, creating pMTL22-*gfp-tet*. Plasmids used for gene replacement were constructed by inserting approximately 2-kb PCR products on each end of the *gfp-tet* marker; these products were obtained from regions directly upstream and downstream of the gene of interest (Table 1) (17). The fragments were amplified with a Perkin-Elmer 9600 thermocycler by using a 20:1 *Taq-Pfu* polymerase mixture (*Taq* was obtained from Pharmacia, and *Pfu* was obtained from Stratagene). The PCR fragment was inserted into pMTL22-*gfp-tet*, yielding plasmid pMTL22-*arcA*-KO. This plasmid was used to transform *H. influenzae* KW20, and clones that were positive for double recombination events were identified by the presence of tetracycline resistance and the absence of ampicillin resistance, which indicated that the chromosomal *arcA* gene was replaced by the *gfp-tet* fragment. *H. influenzae* KW20 recombinants obtained in this way were a source of genomic DNA for transformation of the virulent strain *H. influenzae* ATCC 10211, as described above. Confirmation of the proper insertion of DNA fragments into the *H. influenzae* chromosome was obtained by performing PCR with genomic DNA from the transformed strains. PCR products were obtained by using primers inside the *gfp-tet* genes and directly adjacent to the 2-kb flanking regions originally cloned into pMTL22. Candidate clones were investigated further by digestion analysis of PCR products by using at least two different restriction enzymes to generate fragments for analysis on agarose gels. Sequence information (17) was used to verify the fragment sizes detected.

Complementation of the *arcA* mutation. For complementation studies, the *arcA* genes from both *E. coli* and *H. influenzae* were obtained by PCR by using the oligonucleotides listed in Table 2, and they were sequenced by modified Sanger DNA sequencing. The *arcA* coding sequence from *H. influenzae* is denoted *arcA*Hi, and that from *E. coli* is denoted *arcA*Ec. Both genes were cloned into pJRD215, a broad-host-range mobilizable plasmid (13), and were tested in an *E. coli arcA* mutant bearing an *sdh*::*lacZ* fusion (11). The *sdh* (succinate dehydrogenase) gene of *E. coli* is regulated by ArcA.

The *arcA* gene from *H. influenzae* was inserted into pMTL22. The resulting plasmid, pMTL22-*arcA*-*tetR*, was used to insert the *arcA* coding sequence at its original position in the chromosome. To achieve this, the *arcA* mutant carrying a kanamycin cassette insertion was transformed with pMTL22-*arcA*-*tetR*. Tetracycline-resistant transformants that had lost resistance to kanamycin were investigated further. Correct insertion of the *arcA-gfp-tetR* cassette was confirmed by PCR as described above. The resulting strain was designated Hib3 (Table 3).

Determination of capsule and LOS. For determination of capsule, bacteria were grown aerobically in complete BHI medium to an optical density at 600 nm of 1.0 (2×10^8 CFU/ml), centrifuged, and washed in 10 mM HEPES. The amounts of bacterial cells in the samples were standardized; the cells were resuspended in 10 mM HEPES with 10 mM EDTA and incubated for 1 h at 37°C without shaking (37). Cells were then removed by centrifugation ($5,000 \times g$, 5 min), and the polyribose ribitol phosphate (type b capsular subunit) content of the supernatant was determined by a competitive enzyme-linked immunosorbent assay. Serial dilutions of the supernatants were used in competition with *H. influenzae* type b vaccine conjugate (HibTITER). The original culture supernatants were also analyzed in this assay in order to determine the amounts of free polyribose ribitol phosphate which they contained. The capsule concentration was expressed in absorbance units (measured at 405 nm for the appropriate dilution).

LOS was purified from whole *H. influenzae* cells (31), electrophoresed on a 14% polyacrylamide gel, and silver stained by using a commercial silver staining kit (Bio-Rad).

Assay for classical complement activity. Anti-sheep hemolysin was titrated according to the instructions of the manufacturer (BioMerieux, Lyon, France). Total classical complement pathway hemolysin activity was determined as previously described by Coligan et al. (10).

Assay for serum sample antibody titer. An enzyme-linked immunosorbent assay was performed with plates coated with either whole unencapsulated *H. influenzae* (Hib⁻) cells (61) or capsule antigen (Act-Hib; Pasteur Merieux). Each serum sample was used as a primary antibody (1:5 dilution), and protein A-horseradish peroxidase was used as a secondary antibody. The results were expressed as percentages of the value obtained with positive control Hib antiserum (1:100 dilution; Difco) as the primary antibody. Experiments were done in duplicate.

Phenotype in human serum. Human serum samples were obtained from the Centre de Transfusion Sanguinaire, University of Geneva Hospital, and were between 1 and 6 weeks old, at which time they still retained full complement activity (data not shown). *H. influenzae* strains were grown aerobically at 37°C in BHI medium to the mid-log phase and were inoculated into either undiluted serum or blood at a concentration of 10^6 CFU/ml. The preparations were incubated at 37°C with mild shaking, and viable counts were determined by plating serial dilutions on BHI agar. The bacterial counts obtained from serial dilutions were invariably in the linear range of dilution, suggesting that the killing during growth on plates by codiluted blood or serum components was negligible.

Hep-2 cell adhesion experiments. Hep-2 cells were seeded at a concentration of 1×10^5 cells/well into 24-well culture plates (Costar) and allowed to grow overnight in 1 ml of Dulbecco's modified Eagle medium supplemented with penicillin and streptomycin (Gibco-BRL) at 37°C under a water-saturated 5% CO_2 atmosphere. Before *H. influenzae* cells were added, the Hep-2 cells were washed once with Hanks balanced salt solution (Gibco-BRL) and supplemented with antibiotic-free growth medium. Bacteria were added at a concentration of 10^7 CFU/well. The plates were centrifuged for 5 min at $500 \times g$ to enhance bacterial contact with the cell surface and then were incubated for 1 h at 37°C to allow adherence of bacteria to cell surfaces. The Hep-2 cells were washed twice with Hanks balanced salt solution and stripped from the surfaces of the plates by trypsin-EDTA treatment. The resulting cell suspensions were plated on BHI agar plates, and colonies were counted after incubation at 37°C.

TABLE 2. Oligonucleotides for PCR of the *arcA* gene

Type of primer (plasmid)	*H. influenzae arcA* oligonucleotide (5′-3′)[a]	*E. coli arcA* oligonucleotide (5′-3′)[a]
5′ (pJRD215)	AACCC**AAGCTT**ATGACTACTCCAAAAATTCTC	AACCC**AAGCTT**ATGCAGACCCCGCACATT
3′ (pJRD215)	ACATG**CCATGG**TTATTCAATATCTCCACAAAAAC	ACATG**CCATGG**TTAATCTTCCAGATCACCG
5′ (pMTL22)	GC**TCTAGA**ATGACTACTCCAAAAA	GC**TCTAGA**ATGCAGACCCCGCACA
3′ (pMTL22)	CG**GCTAGC**<u>C</u>ATTGCCTTTCCTCCTTATTCAATATCTCCAC	CG**GCTAGC**<u>C</u>ATTGCCTTTCCTCCTTAATCTTCCAGATCA

[a] Restriction enzyme sites used for cloning are indicated by boldface type. Underlined nucleotides were added to keep the *arcA* gene in frame with upstream genomic sequences.

TABLE 3. *H. influenzae* type b strains used in studies and genotypes

Strain	Genotype	Description
Hib	ATCC 10211	Wild type
Hib1	ATCC 10211/Δ*arcA*::*kan*	Mutant by insertion of *kanR* cassette
Hib2	ATCC 10211/Δ*arcA*::*gfp-tet*	*arcA* gene replacement with *gfp-tetR* cassette
Hib3	ATCC 10211/*arcA*::*gfp-tet*	Repaired mutant *arcA*::*kan* (*arcA* reinserted into normal position in chromosome along with *gfp-tetR* cassette)

Two-dimensional PAGE and proteome analysis. Two-dimensional polyacrylamide gel electrophoresis (PAGE) of lysates of whole bacterial cells was performed as described by O'Farrell (47), with the following modifications: immobilized pH gradient (pH range, 3.5 to 10.0 [sigmoidal] or 4.0 to 7.0) strips were used for the first dimension, and 12.5% PAGE was used for the second dimension. A highly sensitive silver staining protocol (32) was used for protein detection. Lysates were prepared at least twice, and gels were run at least twice for each lysate to ensure reproducibility of the protein staining patterns. The gels were scanned at high resolution, and proteome comparisons were done by using the Melanie II software (1). Gels were also prepared by using a modified silver staining protocol compatible with mass spectrometry (52). When membrane preparations were subjected to two-dimensional PAGE, the samples were solubilized with a combination of urea, thiourea, and Triton X-100 (J.-C. Sanchez, personal communication).

Spots were excised from gels, reduced, alkylated, and digested with unmodified trypsin overnight in a small volume. The resulting solution was used directly for matrix-assisted laser desorption ionization–time of flight mass spectrometry. The resulting peptide masses were used to search a nonredundant database. Triple quadrupole-nanospray mass spectrometry was used to obtain a sequence tag if peptide masses alone were insufficient to correctly identify certain proteins (58).

Total membrane protein preparation and Western blotting. Total membrane fractions of *H. influenzae* cells were prepared with the addition of a freeze-thaw step during spheroplast preparation (59). Total membrane proteins were electrophoresed by performing sodium dodecyl sulfate (SDS)–10% PAGE and were blocked in 2% bovine serum albumin–0.05% Tween 20. Serum samples (1:5 dilution) or a 1:100 dilution of Hib antiserum was used as the primary antibody; protein A coupled to horseradish peroxidase was used as the secondary antibody. Detection was performed with 4-chloro-1-naphthol and H_2O_2.

RESULTS

Construction of *H. influenzae* knockout mutants. We identified the *arcA* coding sequence in the genome sequence of *H. influenzae* Rd by using The Institute for Genome Research microbial database as a query (http://www.tigr.org/tigr-scripts/CMR2/GenomePage3.spl?database=ghi). *arcA* knockout mutants were made either by insertion of a kanamycin resistance cassette, resulting in mutant Hib1 (*arcA*::*kan*), or by nonpolar gene replacement with a green fluorescent protein-tetracycline resistance cassette, resulting in mutant Hib2 (*arcA*::*tet*).

Growth rates of *H. influenzae* mutants in vitro. The doubling times of the two *arcA* mutants constructed in this study, Hib1 and Hib2, the wild-type parent strain, Hib, and a rescued mutant strain, Hib3, under aerobic conditions in complete BHI medium were between 47 and 52 min at 37°C. In MIc minimal medium, the doubling times ranged from 92 to 95 min. Thus, the ArcA phenotype did not appear to cause a significant growth defect in vitro.

Virulence of *H. influenzae arcA* in BALB/c mice. The virulence of the *arcA* knockout strain Hib2 (*arcA*::*tet*) was compared to that of wild-type *H. influenzae* and that of a *tonB* knockout strain constructed in our laboratory in the isogenic
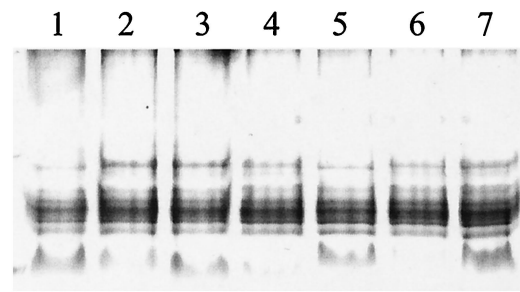

FIG. 1. LOS profiles of the *arcA* mutants and the wild type on 14% PAGE gels. Lane 1, Hib⁻ strain (unencapsulated); lane 2, strain Hib1 (*arcA*::*kan*); lane 3, strain Hib2 (*arcA*::*tet*); lane 4, wild-type strain Hib; lane 5, Hib3 (*arcA-gfp-tet*); lane 6, strain Hib2 after exposure to serum; lane 7, wild-type strain Hib after exposure to serum.

background (results not shown). TonB was previously shown to be important for mouse virulence (27). We found that disruption of the *arcA* gene in *H. influenzae* resulted in a reduction in virulence similar to that found for a *tonB* knockout mutant, and the medium lethal doses for the wild-type parent strain and the *arcA* and *tonB* mutants were 6.4×10^4, 1.0×10^6, and 7.0×10^5 CFU, respectively.

Expression of surface structures (LOS and capsule) in *H. influenzae arcA* mutants. The LOS profiles of the *H. influenzae arcA* mutant and the wild-type parent strain were compared by PAGE. No major differences between the two strains were found after growth on BHI medium or when the organisms were exposed to human serum (Fig. 1). We therefore assumed that ArcA does not influence the synthesis of LOS in *H. influenzae*.

As type b capsule has previously been identified as a major virulence determinant in *H. influenzae* (36, 42), we verified that the *arcA* mutant and wild-type parent strain had similar capsule compositions by using an agglutination assay with a specific anti-*H. influenzae* type b antibody, and we determined the amounts of capsule produced by the two strains. The levels of production of cell-associated polyribose ribitol phosphate (type b capsule) by the *arcA* mutant (175 ± 9 arbitrary units) and the wild type (184 ± 9 arbitrary units) were found to be identical within the expected experimental error; a nonencapsulated strain (61) was used as a control (<10 arbitrary units). We detected negligible amounts of capsule polymer in the culture supernatants of the *arcA* mutant and the wild-type parent strain, consistent with the results of other workers (8, 37). We concluded that the quantities of type b capsule produced by the *arcA* mutant and the wild-type parent strain are equivalent.

Bacterial attachment to epithelial cell monolayers was used as a criterion to compare the adhesive properties of the bacterial envelopes. To do this, bacteria were grown either under aerobic conditions or under anaerobic conditions and were incubated with Hep-2 monolayers. We did not find any significant differences in adherence of the *H. influenzae* wild type and *arcA* mutant to Hep-2 cells, and the adherent fraction represented between 2.5 and 3% of the inoculum.

***H. influenzae arcA* is highly sensitive to serum bactericidal activity.** Antimicrobial factors that limit bacterial growth and survival in fresh blood in vitro may represent some of the most
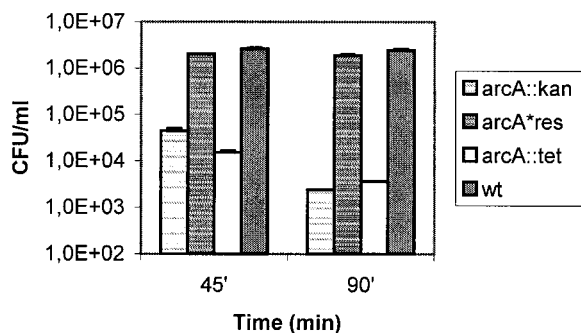
FIG. 2. Bactericidal activity of serum donor 9: survival of *arcA* mutants Hib1 (*arcA*::*kan*) and Hib2 (*arcA*::*tet*), repaired *arcA* mutant Hib3 (arcA*res), and the wild type (wt) in serum from donor 9, expressed in CFU per milliliter. The initial inoculum was 10^6 CFU/ml. At 135 min, the titer of the *arcA* mutants fell below the threshold of detection (100 CFU/ml), while the levels of Hib3 and the wild type remained stable.

potent bactericidal mechanisms during bacterial septicemia as well. However, as human serum is easier to manipulate and is more stable during bacterial culture than whole heparinized blood (results not shown) and contains all complement and antibody components required for bactericidal activity, we decided to use this medium to compare the *H. influenzae* wild type and *arcA* mutants. To compare serum resistance in vitro, bacteria were incubated in serum, and survival was evaluated by measuring the colony-forming ability as a function of incubation time. Figure 2 shows the hypersensitivity of *H. influenzae arcA* strains to human serum compared to the sensitivity of isogenic strains that normally produced the ArcA protein.

In this experiment, the difference in survival between the two *arcA* mutants and the wild type was approximately 1,000-fold after 90 min of incubation. With some serum samples the differences were even greater. The half-lives of each strain in 10 different sera are compared in Table 4. Whereas some of the sera tested showed no bactericidal activity, sera that did reduce bacterial growth and survival eliminated both *arcA* mutants, Hib1 and Hib2, at rates greatly exceeding those at which

both the wild-type parent strain and the *cis*-complemented strain (see below) were eliminated. The observed half-lives demonstrate that the behaviors of the mutants constructed by using a polar (*arcA*::*kan*) or nonpolar (*arcA*::*tet*) gene knockout approach were identical.

Since serum has virtually no source of hemin, which is abundantly available in blood and is necessary for aerobic growth of *H. influenzae*, half-lives were also determined with hemin (10 μg/ml) added to the serum samples. No significant differences in survival compared to survival without hemin were observed, however. The survival of isogenic unencapsulated *H. influenzae* (Hib⁻) strains in serum was very limited. Bacterial titers were no longer detectable after 20 min of serum incubation, underlining the importance of capsule in serum resistance.

***trans* complementation of *arcA* mutations in *E. coli* and *H. influenzae*.** The *H. influenzae arcA* gene was identified only on the basis of its sequence similarity to *E. coli arcA* (78% identity). To show that the two genes were indeed functionally equivalent, they were PCR amplified from the genomic DNAs and introduced into broad-host-range plasmid pJRD215 (13).

Complementation of *E. coli arcA* was demonstrated by using an *sdh*::*lacZ* fusion (11). In *E. coli*, *arcA* represses the expression of succinate dehydrogenase (Sdh) under anaerobic conditions, and complementation of *arcA* in *trans* should thus restore conditional repression of β-galactosidase production in this background. This was indeed observed when either the *E. coli* or *H. influenzae arcA* coding plasmid was introduced into this strain (Fig. 3), showing that *H. influenzae* ArcA is at least partially functional in *E. coli*.

We observed only partial complementation of *H. influenzae arcA* with pJRD215::*arcA*ₕᵢ when serum resistance was used as an arbitrary readout, and no complementation was observed when the homologous *E. coli* gene was supplied in *trans* (Fig. 4). The increased serum sensitivity observed in the *H. influenzae arcA* control strain carrying plasmid pJRD215 remains unexplained and prompted us to abandon this approach.

Rescue of the *arcA* mutation. As an alternative to using genetic complementation in *trans*, we decided to use marker rescue to show that the cloned *arcA* sequences could fully

TABLE 4. Half-lives of *H. influenzae* type b strains, complement activity, and antibody levels of serum samples

Serum	Half-lives (min)[a]				1/CD₅₀[b]	Antibody titers[c]	
	arcA::*tet*	*arcA*::*kan*	Wild type	Repaired *arcA*		Anti-protein	Anti-capsule
1	4.5 ± 0.01	4.5 ± 0.01	16.0 ± 2.4	42.3 ± 5.4	ND[d]	83	48
2	5.2 ± 1.1	6.3 ± 1.2	13.5 ± 1.2	—[e]	30	226	13
3	8.7 ± 1.1	15.4 ± 3.8	29.1 ± 4.9	25.0 ± 4.7	43	74	85
4	5.5 ± 2.0	5.3 ± 1.1	23.6 ± 3.0	9.2 ± 0.6	29	186	124
5	5.2 ± 1.0	9.3 ± 1.0	31.4 ± 1.5	ND	ND	152	86
6	—	—	—	—	21	99	13
7	—	—	—	—	16	81	11
8	8.0 ± 0.5	7.8 ± 0.2	16.0 ± 0.8	14.5 ± 1.3	167	171	23
9	3.7 ± 0.3	9.8 ± 0.5	—	—	70	80	7
10	9.7 ± 1.0	11.0 ± 0.5	39.3 ± 8.4	17.8 ± 0.1	37	123	8

[a] Half-lives ± standard deviations are shown.

[b] 1/CD₅₀ is the inverse of the dilution factor needed to obtain 50% lysis of erythrocyte-antibody complexes (sheep blood erythrocytes and hemolysin). Thus, the higher the value, the stronger the complement activity. The values are the averages of at least two experiments. Measurements were not obtained for sera 1 and 5 because the assay was still in development when the samples passed the 6-week expiration point (see Materials and Methods).

[c] Amounts of anti-protein and anti-capsule antibody expressed as percentages of the control antiserum. The values are the averages of at least two experiments.

[d] ND, not determined.

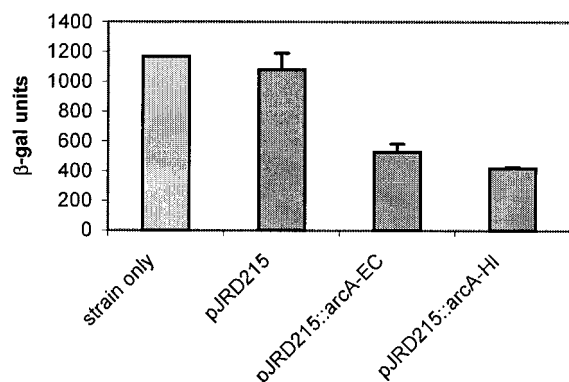[e] The number of CFU per milliliter was constant or increased during the time measured.

FIG. 3. β-Galactosidase activity in the complemented *E. coli arcA* mutant: comparison of *E. coli* strains carrying plasmids for complementing the chromosomal *arcA* mutation in *cis*. The β-galactosidase (β-gal) activity of a succinate dehydrogenase (*sdh*)-*lacZ* reporter fusion was measured during anaerobic growth.

restore the wild-type phenotype to *H. influenzae* strain Hib1. To do this, we constructed suicide plasmids carrying either of the *arcA* genes as a transcriptional fusion to *gfp-tet* and flanked by 2 kb of adjacent *H. influenzae* DNA to enable reciprocal recombination. The *arcA* gene from *H. influenzae* was readily reintroduced into the chromosome of the *arcA*$_{Hi}$::*kan* mutant to create strain Hib3 (see Materials and Methods), which was resistant to tetracycline and sensitive to kanamycin. In Hib3 serum resistance was restored to wild-type levels (Fig. 2 and Table 4). When attempts were made to exchange *arcA*$_{Hi}$::*kan* for the *arcA*$_{Ec}$-*gfp-tetR* coding sequence of *E. coli*, no tetracycline-resistant transformants were obtained. Our inability to construct these recombinants remains unexplained.

Serum factor(s) responsible for *arcA* hypersensitivity. In order to determine the contributions of antibody and complement to the observed serum hypersensitivity of the *H. influenzae arcA* mutants, the bacterial strains were also exposed to heat-treated sera devoid of complement activity. In decomplemented serum, bacterial survival was the same for mutant and

wild-type strains; the *H. influenzae* titers remained constant or increased slightly during the first few hours of incubation (results not shown). We concluded, therefore, that at least one heat-labile serum factor was involved in the enhanced bactericidal activity directed against the *H. influenzae arcA* mutants. Although this factor is likely to be complement (6, 55), we cannot rule out the possibility that some other heat-labile factor might play a role in *H. influenzae* serum killing or the possibility that antibody is required as well.

Assay of classical complement activity and antibody titer of serum samples. In an attempt to correlate the *arcA*-specific bactericidal activity of human serum with either classical complement activity or antibody titers, we determined both these parameters in each of the serum samples tested. The complement activities for eight serum samples (Table 4) showed a correlation with the half-lives of *arcA* and wild-type strains, and the threshold level of complement activity was around 30; below this level no killing of the *arcA* mutants was observed. No correlation between anti-*H. influenzae* antibody titers and *arcA*-specific bactericidal activity could be inferred from the data obtained. Antibody titers were measured separately against capsule and protein antigens, but we did not analyze the antigen specificity in each of the serum samples further.

In an attempt to estimate the contribution of anti-Hib antibody to serum bactericidal activity, we exposed wild-type and mutant bacteria to mixed serum samples in which the total combined anti-Hib antibody titer varied in the context of constant complement activity. Although we observed that killing of both mutant and wild-type bacteria correlated positively with antibody titers, higher antibody levels did not change the sensitivity of the *arcA* mutant compared with the sensitivity of the wild-type strain. The role of complement in bactericidal activity was well illustrated by comparison of sera 6, 7, and 9 (Table 4). Whereas the antibody titers for sera 6, 7, and 9 were quite similar, serum 9 had a much higher classical complement activity. Thus, antibody contributed equally to elimination of wild-type and mutant bacteria from serum, whereas complement activity appeared to discriminate between the two strains.

Analysis of the ArcA regulon of *H. influenzae*. We set out to identify proteins regulated by ArcA, including possible virulence factors involved in serum resistance, by using two-dimensional gel electrophoresis. In *E. coli*, ArcA regulates the expression of a considerable number of proteins, including L-lactate dehydrogenase, superoxide dismutase, cytochrome *d* oxidase, and succinate dehydrogenase (39). Expression of some ArcA-regulated proteins, such as cytochrome *d* oxidase, is induced under anaerobic growth conditions, whereas most of the other proteins are repressed under anaerobic growth conditions. We compared *H. influenzae* protein profiles under both of these growth conditions to identify genes regulated by ArcA. Silver-stained two-dimensional gels of total cell lysates from mutant and wild-type strains grown aerobically in minimal medium showed differences too minor to be analyzed by mass spectrometry. However, the impact of the *arcA* mutation on global gene expression was evident when the protein profiles of anaerobically grown bacteria were compared (Fig. 5). In this analysis, the *arcA* mutant profile contained many protein spots that either were totally absent from the profile of the wild-type parent strain or had much lower intensities, illustrating the size of the *arcA* regulon in *H. influenzae*. In addition, the wild-type
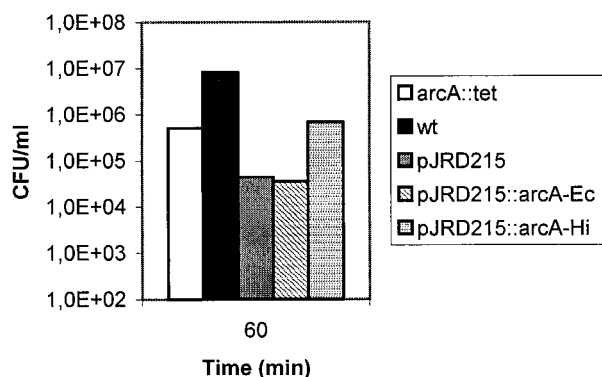

FIG. 4. Comparison of bactericidal activities against *arcA* strains carrying the pJRD215 and pJRD215::*arcA* plasmids. The *arcA* mutant, the wild-type strain (wt), and the *arcA* mutant complemented with plasmid-coded *arcA* were inoculated at a concentration of 10^7 CFU/ml into serum. After 1 h of incubation at 37°C, the numbers of CFU were determined by serial dilution (standard error, less than 5%).

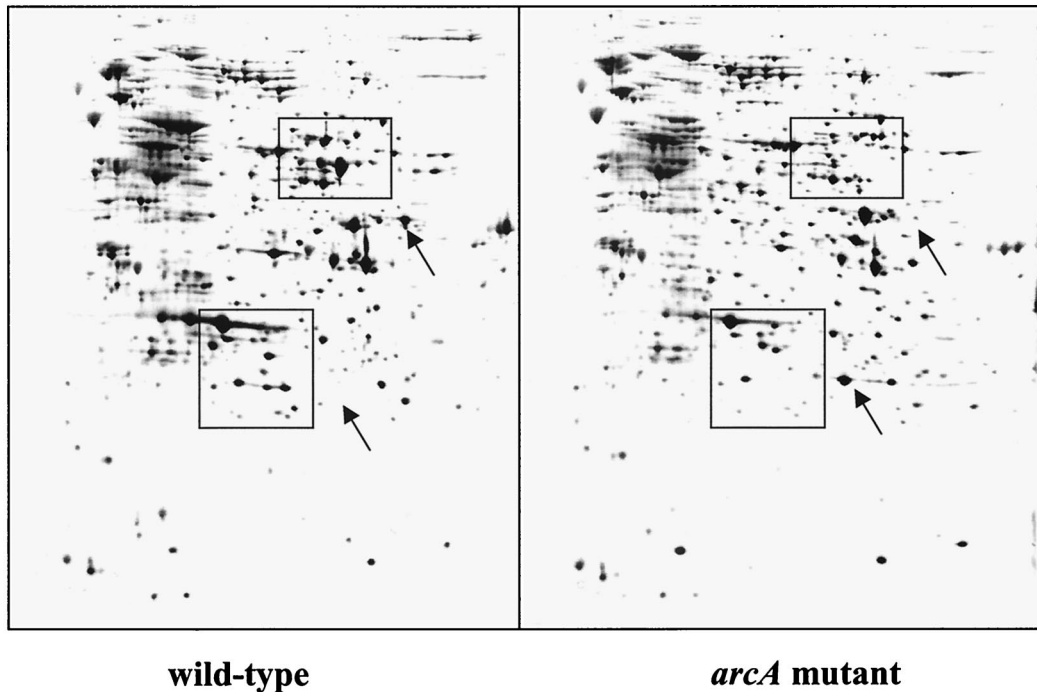**wild-type**   *arcA* **mutant**

FIG. 5. Wild-type strain Hib and *arcA* mutant proteomes for total cell lysates after anaerobic growth. Regions with many differences are enclosed by boxes. The arrows indicate some of the major differences.

parent strain profile contained a number of spots that either were absent or had low intensities in the *arcA* mutant profile. Although total cell lysates were used as samples for two-dimensional gel electrophoresis, proteins with very high pI values (pI >10), as well as many hydrophobic or membrane proteins, could not be separated properly in the first dimension and thus were poorly represented on the gel. We were especially interested in identifying differences in expression of membrane proteins, as these molecules might be primary targets for complement-mediated killing.

Membrane fractions. In view of the intrinsic difficulties in analyzing bacterial membrane proteins on two-dimensional gels and the lower complexity of these protein fractions, we decided to compare membrane protein expression data by using one-dimensional SDS-PAGE. Total membrane fractions were prepared from the wild-type and mutant strains grown under aerobic conditions (Fig. 6) and anaerobic conditions (Fig. 7) and were compared by using one-dimensional SDS-PAGE. The wild-type parent strain and the *cis*-complemented mutant strain were indistinguishable, as were the two mutant strains. However, a number of differences were observed between *arcA* knockout mutants on the one hand and ArcA proficient strains on the other. Three membrane-associated proteins were identified by mass spectrometry (see Materials and Methods) as fumarate reductase, formate dehydrogenase, and L-lactate dehydrogenase. All three of these proteins are known cytoplasmic membrane-associated proteins involved in either cellular respiration or fermentation.

Formate dehydrogenase (SwissProt accession no. P46448), which allows the bacteria to use formate as a unique electron donor when oxygen is not present, was detected at the ex-
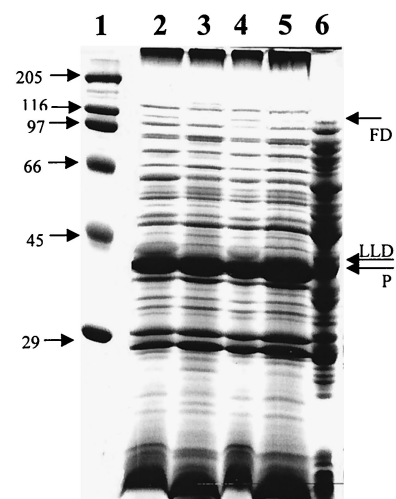

FIG. 6. Aerobically expressed total membrane proteins. Lane 1, molecular mass standard (the molecular masses of the proteins in the standard, in kilodaltons, are indicated on the left); lane 2, strain Hib1 (*arcA*::*kan*); lane 3, strain Hib3 (*arcA-gfp-tet*); lane 4, strain Hib2 (*arcA*::*tet*); lane 5, wild-type strain Hib; lane 6, whole-cell lysate. Membrane proteins were prepared, as described in Materials and Methods, from cells grown aerobically in BHI medium. It was difficult to separate the cytoplasmic and outer membrane fractions, probably due to the high capsule content. Therefore, the gels represent the total membrane fraction. The most reproducible differences were the differences in two proteins, formate dehydrogenase (FD) (115 kDa) and L-lactate dehydrogenase (LLD) (42 kDa). Other small differences were observed but were not reproducible. The probable position of porin (P) is also indicated.
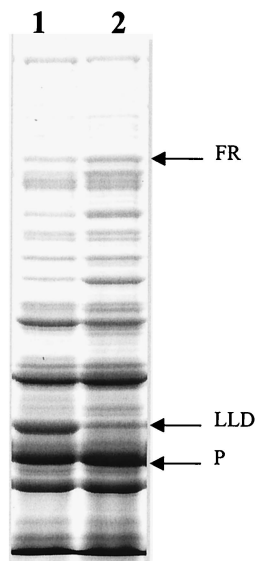
FIG. 7. Anaerobically expressed membrane proteins. Total membrane proteins of anaerobically grown Hib2 (*arcA::tet*) (lane 1) were compared with membranes prepared from wild-type *H. influenzae* (lane 2). Membrane proteins were prepared, as described in Materials and Methods, from bacteria grown anaerobically in BHI medium supplemented with KNO_3 (0.5%). The gel was electrophoresed longer in order to better resolve higher-molecular-weight bands. Among the several differences observed, differences in two proteins were identified; fumarate reductase (FR) (66 kDa) was more highly expressed in ArcA+ strains, and L-lactate dehydrogenase (LLD) (42 kDa) was derepressed in ArcA– strains. The probable position of porin (P) is also indicated.

pected position in the two *H. influenzae arcA* mutants but was absent in the wild type and in the *cis*-complemented *arcA* mutant grown under aerobic conditions (Fig. 6). Thus, similar to the *E. coli* enzyme, this enzyme is repressed under aerobic conditions in *H. influenzae* and is derepressed during anaerobiosis (39), yet the expression of formate dehydrogenase in *E. coli* appears to be controlled by the regulator Fnr, not ArcA (39).

Fumarate reductase (SwissProt accession no. P44894) is also regulated by Fnr in *E. coli* (39). Fumarate reductase interacts with several electron donors and helps maintain the proton motor force during anaerobiosis. We observed expression of this enzyme in the wild-type *H. influenzae* strain but not in the *arcA* mutants when the organisms were grown under anaerobic conditions (Fig. 7).

In *E. coli*, ArcA represses L-lactate dehydrogenase (SwissProt accession no. P46454) only under anaerobic growth conditions (19). Interestingly, in *H. influenzae* we observed ArcA-mediated repression of L-lactate dehydrogenase under both anaerobic and aerobic growth conditions (Fig. 6 and 7). This suggests not only that ArcA exerts both positive and negative regulation but also that it may do so in oxygen-dependent and oxygen-independent ways.

DISCUSSION

In vivo passage of *H. influenzae* from mucosal colonization sites in the upper respiratory tract to the general circulation and then the spinal fluid requires a series of physiological adaptations by the pathogen. *H. influenzae* must cope with changes in oxygen availability, osmolarity, and nutrient availability, as well as exposure to antimicrobial mechanisms, such as defensins, complement, and other mediators of innate and adaptive immunity. We hypothesized that two-component regulatory systems, such as ArcAB, dedicated to the detection of environmental signals and consequent fine-tuning of the bacterial physiology might play important roles in the virulence of *H. influenzae*.

Role of ArcA in serum resistance. The *arcA* gene, as identified in the *H. influenzae* KW20 genome sequence, was found to functionally complement an *E. coli arcA* mutant and rescue an *H. influenzae arcA::kan* knockout mutant, restoring wild-type protein expression profiles and serum resistance. *H. influenzae arcA* mutants grew normally in vitro, and the production of capsule and LOS seemed to be unaffected. This was an important observation because it suggested that an as-yet-unidentified factor might be responsible for the observed reduction in mouse virulence. Taking into consideration the fact that animal models for the virulence of *H. influenzae*, a strictly human pathogen, have only limited predictive value with respect to bacterial infection in humans, we set out to look for interactions of wild-type and mutant *H. influenzae* strains with human factors like the epithelial cell surface, professional phagocytes (results not shown), and human blood and serum. Apart from the changes observed in protein expression profiles, the most striking phenotype associated with the loss of functional ArcA was the strongly increased sensitivity to bactericidal activities in human blood and serum.

Since the introduction of a vaccine directed against (conjugated) type b capsule, the incidence of bacterial meningitis caused by *H. influenzae* has dramatically decreased (21), illustrating the key role of specific immunity in clearance of the bacterium from the circulation. Although the relative contributions of antibody, complement, and phagocytes in protection against *H. influenzae* serotype b septicemia and meningitis have not been clearly established, it has been well established that opsonizing antibodies stimulate phagocytosis and bactericidal activity (4, 30, 45) and that the potency of the latter is dependent on the presence of complement (50).

We show here that a mutant *H. influenzae* type b strain has markedly increased serum sensitivity and that the increased susceptibility to serum bactericidal effects involves a heat-labile serum factor. While the results obtained with 10 human sera strongly suggest that the *arcA* mutant has increased sensitivity to antibody-mediated complement killing, we cannot yet rule out the possibility that another heat-labile factor, such as bactericidal permeability-increasing protein (BPI), determines the high bactericidal activity against the *arcA* mutants. BPI has been shown to mediate killing of certain gram-negative bacteria by binding to lipopolysaccharide and causing growth arrest, damage to the inner membrane, and inhibition of all lipopolysaccharide-induced host cell responses (15, 48). This protein also seems to have an opsonizing activity (33). We think that it is unlikely that BPI plays a role because no differences in LOS between *arcA* and *arcA*+ strains were found.

In these experiments, no bactericidal activities could be attributed to antibody alone. There was no clear-cut relationship between antibody titer and bactericidal activity in the presence

of complement, although high antibody titers appeared to stimulate the bactericidal activities of sera. This suggests a helper role for antibody to induce efficient complement-mediated killing and that *arcA* mutants are markedly more sensitive to this mechanism than the wild-type strain.

It is of considerable significance that we observed a difference in serum sensitivity between the ArcA$^+$ and ArcA$^-$ strains in all sera with bactericidal activity. The absence of large variations in the bacterial half-lives in sera with complement activities above the threshold level of 30 may reflect the fact that the quantity of complement itself is no longer rate limiting under these conditions. Nonencapsulated *H. influenzae* was virtually instantaneously eliminated from serum, underlining the protective role of the (type b) capsule, which may well be due to its inhibition of complement activity as well (29). The observed increase in sensitivity to human sera may partially explain the reduced virulence of *arcA* mutants in the mouse septicemia model. However, the mice used were inbred and had not been previously exposed to *H. influenzae* antigens. In view of the results presented here, the virulence model might be improved by priming a B-cell response with defined *H. influenzae* antigens prior to challenge. Additional important experiments include exposing the *arcA* mutant to purified complement factors and knocking out of some of the envelope components in order to further dissect the *arcA* serum hypersensitivity phenotype.

ArcA-regulated proteins. In an attempt to identify serum resistance or serum susceptibility factors in *H. influenzae* type b that might be responsible for the observed *arcA* phenotype, we carefully investigated the compositions of the envelope fractions of the *arcA* mutants and the wild-type parent strain. Previous reports had identified outer membrane proteins P1, P2, P4, and P6 and fimbriae as candidate antibody- or complement factor C3-binding factors (20, 23, 24, 29, 41, 43, 44, 56, 60; M. K. Gutierrez, L. S. Joffe, L. J. Forney, and M. P. Glode, Program Abstr. 30th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 41, p. 93, 1990) that might be differentially expressed in the *arcA* background. However, we did not find any significant differences in expression of envelope components between the wild-type and mutant strains, except for a few cytoplasmic membrane enzymes discussed below.

The two-component regulatory system, ArcAB, has been characterized in detail in *E. coli* (34). The wealth of information concerning *E. coli* is only partially helpful in the context of *H. influenzae* due to important metabolic differences with *E. coli*, as revealed by genome sequencing (17). Analysis of the *H. influenzae* genome shows that certain genes important for energy production (e.g., components of the tricarboxylic acid cycle, like succinate dehydrogenase, as well as certain *pts* enzymes) are absent compared to the pathways known in *E. coli* (54). In *E. coli*, ArcA is activated by ArcB during the transition from aerobic to anaerobic growth. It then affects the expression of two sets of genes: the genes repressed by ArcA, including the genes encoding tricarboxylic acid cycle and glyoxylate cycle enzymes, SodA, and cytochrome *o* oxidase (all key elements of aerobic metabolism); and the genes activated by ArcA, including the gene for cytochrome *d* oxidase, *pfl* genes (25), and genes that code for enzymes that open the electron transport chain to electron donors other than molecular oxygen. It has been suggested that *H. influenzae* depends mostly

on anaerobic metabolism, particularly when establishing bacteremia (14, 54).

We have started to compare ArcA-regulated genes in *H. influenzae* with their counterparts in *E. coli* and have concentrated first on genes encoding membrane components, anticipating that these might help us explain the serum sensitivity phenotype of the *arcA* mutants. ArcA seems to be responsible for the oxygen-dependent regulation of formate dehydrogenase and fumarate reductase in *H. influenzae*, yet in *E. coli* expression of the corresponding genes appears to be controlled by Fnr, not by ArcA. In addition, Fnr in *E. coli* directly enhances ArcA expression (11), making it difficult to determine the regulatory roles of Fnr and ArcA in global oxygen-dependent gene regulation (39). *H. influenzae* Fnr exhibits 79% sequence identity with Fnr of *E. coli* (as determined with The Institute for genome Research database BLAST) and would be expected to be fully functional, but we have not investigated its physiological role in *H. influenzae*. The physiological role of L-lactate dehydrogenase in *H. influenzae* is also brought into question since this enzyme is expressed under both aerobic and anaerobic conditions (Fig. 6 and 7), in contrast to the situation in *E. coli*. While we were able to identify a number of ArcA-regulated membrane proteins, the full ArcA regulon was not analyzed in detail. The number of proteins regulated directly or indirectly by ArcA seems to be more than 40, as judged from the proteome comparisons (Fig. 5).

Taken together, the results presented here provide an appreciation of the complexity of the *arcAB* regulon in *H. influenzae*. *arcA* knockout mutants grow surprisingly well under both aerobic and anaerobic conditions, in spite of the fact that the expression of a large number of genes is deregulated. The involvement of at least one of the ArcA target genes in complement-mediated serum resistance of *H. influenzae*, in a mechanism that does not seem to involve modifications of either capsule or LOS, is a new finding that may lead to identification of new virulence factors in this species.

The molecular mechanism of complement-mediated killing of gram-negative bacteria is thought to involve serum complement component C3 (46) and the complement membrane attack complex (MAC) inserted into the bacterial outer membrane. The MAC causes a loss of inner membrane potential and inhibits inner membrane respiration (12), which is believed to cause cell death. There is indirect evidence that complement-treated cells try to escape MAC killing by utilizing ATP as an energy source to reestablish a K$^+$ concentration gradient over the cytoplasmic membrane (35). We recently isolated extragenic suppressors of the *arcA* serum sensitivity phenotype, one of which is involved in ATP regeneration (unpublished results), suggesting that this mechanism might also play a role in *H. influenzae* resistance to serum factors. We hypothesize that the metabolic state of *arcA* mutants may not be adequate to resist the MAC. This could be caused by the lack of proteins important for the utilization of electron donors other than molecular oxygen and by the fact that the bacterium consequently has difficulties in making the switch between aerobic and anaerobic physiologies. Thus, the *arcA* mutants may succumb more easily to complement attack in human serum, as well as in the bloodstream of the mouse.

With recent improvements in separation and detection techniques (22, 28, 49), regulon mapping is becoming feasible by

using proteomics as a tool. Thus, exhaustive identification of *arcA*-regulated genes in *H. influenzae* has become a realistic option. Clearly, we need to understand more about respiration and gene regulation in *H. influenzae*. This should provide information about just how comparable these processes are in gram-negative bacteria and in what ways these processes affect pathogenicity.

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