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Chromosomal Expression of the *Haemophilus influenzae* Hap Autotransporter Allows Fine-Tuned Regulation of Adhesive Potential via Inhibition of Intermolecular Autoproteolysis

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The *Haemophilus influenzae* Hap autotransporter is a nonpilus adhesin that promotes adherence to respiratory epithelial cells and selected extracellular matrix proteins and facilitates bacterial aggregation and microcolony formation. Hap consists of a 45-kDa outer membrane translocator domain called Hap_β and a 110-kDa extracellular passenger domain called Hap_S. All adhesive activity resides within Hap_S, which also contains protease activity and directs its own secretion from the bacterial cell surface via intermolecular autoproteolysis. In the present study, we sought to determine the relationship between the magnitude of Hap expression, the efficiency of Hap autoproteolysis, and the level of Hap-mediated adherence and aggregation. We found that a minimum threshold of Hap precursor was required for autoproteolysis and that this threshold approximated expression of Hap from a chromosomal allele, as occurs in *H. influenzae* clinical isolates. Chromosomal expression of wild-type Hap was sufficient to promote significant adherence to epithelial cells and extracellular matrix proteins, and adherence was enhanced substantially by inhibition of autoproteolysis. In contrast, chromosomal expression of Hap was sufficient to promote bacterial aggregation only when autoproteolysis was inhibited, indicating that the threshold for Hap-mediated aggregation is above the threshold for autoproteolysis. These results highlight the critical role of autoproteolysis and an intermolecular mechanism of cleavage in controlling the diverse adhesive activities of Hap.

Most bacterial diseases begin with colonization of a particular mucosal surface. Successful colonization requires that bacteria overcome mechanical cleansing and evade the local immune response. A number of specific mechanisms exist to achieve these goals, including the expression of surface-exposed proteins called adhesins, which bind to receptors on the host epithelial surface, and the formation of structured communities called biofilms, which resist mechanical, chemical, and immunological attack (20).

Nontypeable *Haemophilus influenzae* is a nonencapsulated, gram-negative bacterium that commonly causes human respiratory tract disease, including otitis media, sinusitis, bronchitis, and pneumonia (19). Infection by nontypeable *H. influenzae* begins with colonization of the nasopharynx (10). In most individuals, colonization persists for weeks to months without symptoms. However, under certain circumstances bacteria spread contiguously to the middle ear, the sinuses, or the lungs, resulting in disease. Studies of tympanostomy tubes from patients with recurrent otitis media and examination of experimentally infected chinchillas suggest that biofilm formation may represent an important mechanism of persistence by nontypeable *H. influenzae* (3, 11).

To facilitate colonization, *H. influenzae* elaborates both pilus and nonpilus adhesins. The Hap protein is a nonpilus adhesin that was first identified based on its ability to promote intimate interaction with cultured epithelial cells (16). In addition, Hap facilitates adherence to fibronectin, laminin, and collagen IV (5) and mediates bacterial aggregation and microcolony formation, a possible precursor to biofilm formation (7). Hap is a member of the autotransporter family of proteins and consists of an amino-terminal signal sequence, a 110-kDa passenger domain called Hap_S, and a 45-kDa translocator domain called Hap_β (6). As with other autotransporters, the translocator domain is believed to insert into the outer membrane and form a channel through which the passenger domain is extruded to reach the cell surface (6). The Hap_S passenger domain harbors all adhesive activity and is responsible for Hap-mediated adherence and aggregation (4, 7). In addition, Hap_S harbors serine protease activity, which directs autoproteolysis and release of the Hap_S domain into the culture supernatant (4, 6). Mutation of the active site serine residue at position 243 to an alanine (HapS243A) eliminates autoproteolysis and results in accumulation of Hap_S on the bacterial surface (6).

At first glance, Hap autoproteolysis and the resulting release of Hap_S from the bacterial surface seem counterproductive to successful colonization. However, in a recent study, we found that Hap autoproteolysis occurs by an intermolecular mechanism dependent on the density of Hap precursor present on the bacterial cell surface (4). Thus, in bacteria expressing low levels of Hap, it is possible that autoproteolysis is minimal and that the quantity of cell-associated Hap_S is sufficient to promote significant interaction with the host epithelium. In additional work, we discovered that autoproteolysis is inhibited by physiologic concentrations of secretory leukocyte protease inhibitor (SLPI), a component of respiratory secretions that is upregulated in the setting of inflammation. In experiments with bacteria expressing wild-type Hap from a multicopy plas-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or description[a]	Reference or source
Strains		
E. coli DH5α	φ80d*lacZ*ΔM15 Δ*lacU169 deoR recA endA1*	Life Technologies
H. influenzae		
N187	Nontypeable strain from which *hap* was originally cloned	St. Geme et al. (16)
Rd	Laboratory strain, capsule-deficient serotype d	Setlow et al. (15)
DB117	Derivative of Rd with *rec-1* mutation	Setlow et al. (15)
Rd/wt-Hap	Rd with *hap* allele from N187	This study
Rd/HapS243A	Rd with *hap* allele from N187 containing S243A mutation, eliminating Hap autoproteolysis	This study
Plasmids		
pUC19	Amp^r	New England Biolabs
pGJB103	*E. coli-H. influenzae* shuttle vector, Tc^r	Tomb et al. (18)
pJS106	pGJB103 with a 6.7-kb *Pst*I fragment containing *hap*	St. Geme et al. (16)
pDH101::HapS243A	pUC19 with a 6.7-kb *Pst*I fragment containing *hap* with S243A mutation	Hendrixson et al. (6)
pHapS243A	pJS106 with S243A mutation	Hendrixson et al. (6)
pDH101::HapS243A-Cam	pDH101::HapS243A with 1-kb insert containing Cm^r cassette	This study
pN187::Tn*10-4′*	pGJB103 with 9.5-kb insert containing *hap* downstream of Kn^r cassette from transposon Tn*10*	St. Geme et al. (16)
pJS106::Trc	pJS106 with 1.6-kb insert containing *lacI*^{q} and *trc* promoter	Fink et al. (4)

[a] Amp^r, ampicillin resistance, Tc^r, tetracycline resistance; Cm^r, chloramphenicol resistance; Kn^r, kanamycin resistance.

mid, pretreatment with SLPI results in increased surface-associated Hap_S and increased Hap-mediated adherence and aggregation (7). Thus, during natural infection, *H. influenzae* may exploit local concentrations of SLPI to potentiate Hap adhesive activity and initiate formation of biofilms.

In the present study, we examined the relationship between chromosomal expression of Hap and Hap-mediated adherence and aggregation. We found that the level of Hap associated with a chromosomal allele is just above the threshold for intermolecular cleavage, resulting in minimal autoproteolysis and allowing significant adherence to epithelial cells and extracellular matrix. In addition, we discovered that inhibition of Hap autoproteolysis results in small increases in surface-associated Hap and substantial increases in Hap-mediated adherence. Finally, we established that the threshold for Hap-mediated aggregation is achieved only with inhibition of autoproteolysis. Together, these results demonstrate that two different threshold levels of Hap exist, one for autoproteolysis and low- to moderate-level adherence and a second for high-level adherence and aggregation. Transition from the first to the second threshold is achieved when SLPI is present.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in the present study are listed in Table 1. *H. influenzae* strain DB117 was derived from strain Rd and contains a mutation in the *rec-1* allele (15). Both DB117 and Rd contain a *hap* gene with a nonsense mutation at codon 710 and thus produce no stable Hap. *H. influenzae* strains were grown in brain heart infusion (BHI) broth supplemented with NAD and hemin (BHIs) or on BHIs agar or chocolate agar as described previously (1) and were stored at −80°C in BHI broth with 20% glycerol. *Escherichia coli* DH5α was grown on Luria-Bertani (LB) agar or in LB broth. *E. coli* strains were stored at −80°C in LB broth with 50% glycerol. Antibiotic concentrations for *H. influenzae* included tetracycline at 5 μg/ml, chloramphenicol at 2 μg/ml, and kanamycin at 25 μg/ml. *E. coli* strains were grown with chloramphenicol at 25 μg/ml, as appropriate.

Strain Rd/wt-Hap was created by transforming *H. influenzae* strain Rd with linearized pN187::Tn*10-4′* (16), a plasmid harboring a kanamycin resistance cassette ca. 650 bases upstream of the *hap* gene from strain N187. Kanamycin-resistant transformants were screened for allelic exchange and expression of the N187 Hap protein by immunoblot analysis of outer membrane proteins with antiserum N187B (6).

To create strain Rd/HapS243A, a 0.9-kb fragment containing a chloramphenicol resistance cassette was first amplified from pACYC184 with primers designed to introduce *Cla*I sites at each end. The fragment was digested with *Cla*I and ligated into *Cla*I-digested pDH101::HapS243A, inserting the chloramphenicol resistance cassette 600 bases upstream of an N187 *hap* allele possessing a point mutation at codon 243, resulting in conversion of serine to alanine at amino acid 243. The resulting plasmid, pDH101::HapS243A-Cam, was linearized by digestion with *Xmn*I and used to transform *H. influenzae* strain Rd. Expression of HapS243A in chloramphenicol-resistant transformants was confirmed by immunoblot analysis of outer membrane and culture supernatant proteins with guinea pig antiserum GP74, which was raised against purified Hap_S. Allelic exchange in transformants was confirmed by PCR and nucleotide sequencing.

Recombinant DNA methods. DNA ligations, restriction endonuclease digestions, and gel electrophoresis were performed according to standard techniques (14). Plasmids were introduced into *E. coli* strain DH5α by chemical transformation (14). *H. influenzae* strains DB117 and Rd were transformed by using the MIV method of Herriott et al. (8).

Analysis of bacterial culture supernatant and outer membrane fractions. Derivatives of strains DB117 and Rd were grown to an optical density at 600 nm (OD_{600}) of 0.8. Sarkosyl-insoluble outer membrane proteins were isolated by the method of Carlone et al. (2), and extracellular proteins were precipitated from culture supernatants with 10% trichloroacetic acid as described previously (16). Outer membrane fractions were resuspended in 25 μl of 10 mM HEPES (pH 7.4) plus 25 μl of 2× Laemmli buffer, whereas precipitated extracellular proteins were resuspended in 10 μl of 1 M Tris (pH 9.0) plus 10 μl of 2× Laemmli buffer. Protein samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% polyacrylamide gels (9). To ensure that comparable amounts of protein were analyzed, similar volumes from cultures of similar density were loaded into each lane. Resolved proteins were electrotransferred to a nitrocellulose membrane and then detected by immunoblot analysis with antiserum Rab730 diluted 1:500 (6), antiserum RabK2 diluted 1:1,000 (7), antiserum N187B diluted 1:500 (6), or antiserum GP74 diluted 1:2,000, as indicated. An anti-rabbit or anti-guinea pig immunoglobulin G antiserum conjugated to horseradish peroxidase (Sigma) was used as the secondary antibody, and detection of antibody binding was accomplished by incubation of the membrane in a chemiluminescent substrate solution (Pierce) and exposure to film. Quantification of protein band intensities was accomplished by scanning densitometry as described previously (4), normalizing for the quantity of outer membranes loaded in each lane as assessed by determining the levels of major outer membrane protein P4.

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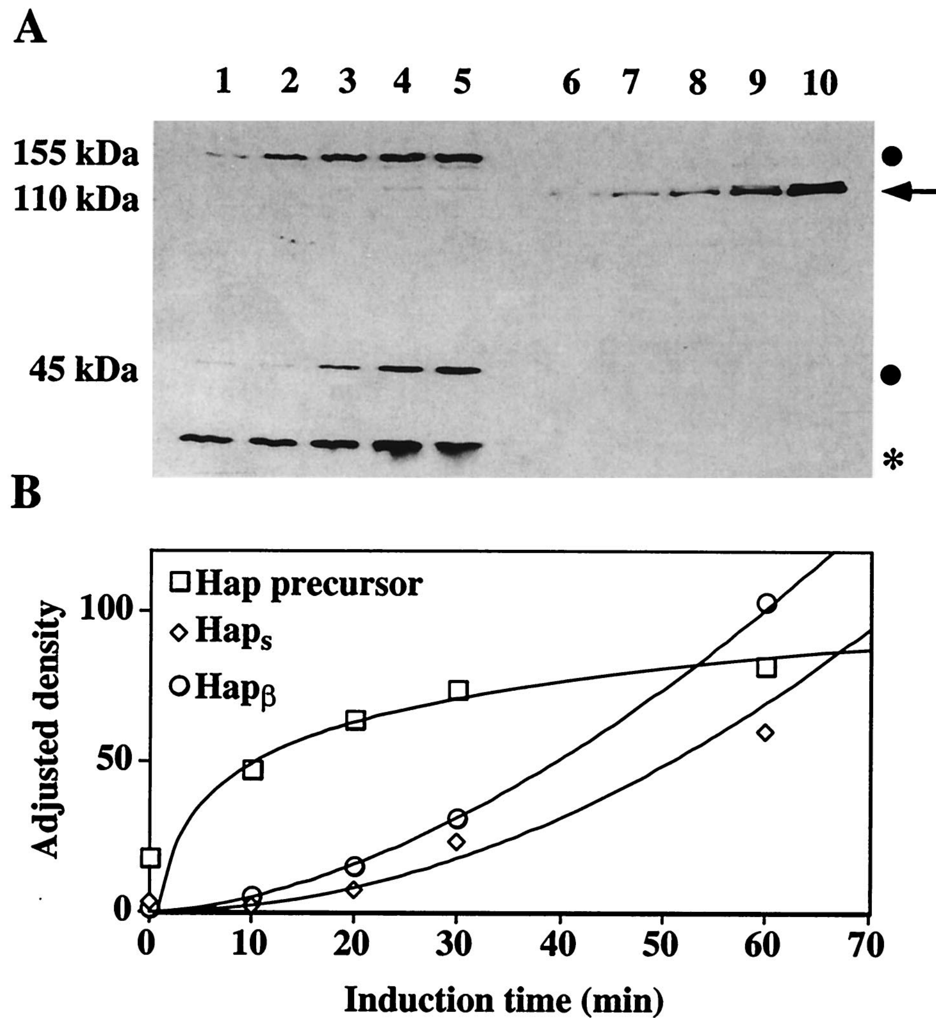

FIG. 1. Relationship between Hap expression levels and the rate of Hap autoproteolytic processing. (A) Analysis of outer membrane proteins (lanes 1 through 5) and culture supernatant proteins (lanes 6 through 10) from DB117/pJS106::Trc99 (containing wild-type Hap expressed under the control of an IPTG-inducible promoter). Cultures were grown to late exponential phase with induction by IPTG for 0 min (lanes 1 and 6), 10 min (lanes 2 and 7), 20 min (lanes 3 and 8), 30 min (lanes 4 and 9), or 60 min (lanes 5 and 10). Proteins were assessed by immunoblot with antiserum Rab730, which reacts with Hap precursor, Hap$_S$, and Hap$_\beta$, and antiserum EPR5-2.1, which reacts with outer membrane protein P4. The dots indicate the 155-kDa Hap precursor protein and the 45-kDa Hap$_\beta$ species resulting from cleavage at the preferred site. The arrow indicates the 110-kDa secreted Hap$_S$ protein. The asterisk indicates outer membrane protein P4, which was included as a loading control. (B) Kinetic analysis of Hap expression and autoproteolytic processing. The intensities of the protein bands shown in panel A were quantified by scanning densitometry to approximate the amounts of Hap precursor and Hap$_\beta$ present in outer membrane fractions and the amounts of Hap$_S$ present in culture supernatants at each time point. The figure plots levels of Hap precursor (□), Hap$_\beta$ (◇), and Hap$_S$ (○). Band intensities of Hap species were normalized by using the intensity of the P4 band at the corresponding time points.

Dot immunoblot analysis of Hap surface expression on intact whole bacteria. Bacteria were grown to late exponential phase, washed once in phosphate-buffered saline (PBS), and then fixed in PBS plus 4% paraformaldehyde for 30 min at room temperature. After one more wash in PBS, bacteria were resuspended in PBS to an OD_{600} of 1.0, and 50 μl of the suspensions were inoculated into wells of a 96-well dot blot manifold apparatus holding a nitrocellulose membrane. Samples were incubated for 30 min and then pulled through the filter by vacuum suction. After blocking for 1 h with Tris-buffered saline plus 5% skim milk, surface-exposed Hap$_S$ was detected with guinea pig antiserum GP74. The signal intensity was quantified by using the NIH Image 1.62 program.

Quantitative adherence assays. Adherence assays with A549 respiratory epithelial cells (ATCC CCL 185) and Chang conjunctival epithelial cells (Wong-Kilbourne derivative, clone 1-5c-4) were performed as described previously (17). A549 cells were maintained in minimum essential medium (MEM-Eagle) with 10% heat-inactivated fetal calf serum. Chang cells were maintained in MEM-Eagle with 10% heat-inactivated fetal calf serum and 1% nonessential amino acids. In assays examining adherence to extracellular matrix proteins, wells of 24-well tissue culture plates precoated with human plasma fibronectin or murine collagen IV (BD Biosciences) were rehydrated in 0.5 ml of MEM (Sigma) at 37°C for 1 h in a tissue culture incubator infused with 5% CO_2. Incubation of strains with 8.5 μM SLPI in assays examining inhibition of autoproteolysis was performed as described previously (7). The percent adherence was calculated by dividing the number of adherent CFU per monolayer by the number of inoculated CFU. Mean levels of adherence were compared for statistical significance by using a two-tailed Student *t* test, when appropriate.

Quantitative aggregation assays. Bacteria were grown to late exponential phase, and cultures were then set standing at room temperature for 4 h while taking OD_{600} readings at 30-min intervals.

IPTG induction of Hap expression. DB117/pJS106::Trc was resuspended into 50 ml of BHIs to an initial OD_{600} of 0.2. The culture was split equally into five tubes, which were incubated at 37°C to late exponential phase (ca. 2 h). IPTG (isopropyl-β-D-thiogalactopyranoside; 0.1 mM final concentration) was added to successive culture tubes at 10, 20, 30, and 60 min prior to terminating the growth.

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No IPTG was added to the fifth culture tube. Completed cultures were split into fractions for analysis by dot immunoblot, quantitative aggregation in standing culture, quantitative adherence to Chang cells, and SDS-PAGE immunoblot of outer membrane and culture supernatant proteins, as described above.

RESULTS

Hap autoproteolysis requires a threshold amount of Hap precursor in the bacterial outer membrane. In previous work, we presented evidence that Hap autoproteolysis occurs via an intermolecular mechanism that depends on the density of Hap precursor present on the bacterial cell surface (4). To confirm our earlier observations, we exploited *H. influenzae* strain DB117/pJS106::Trc, which expresses Hap from a multicopy plasmid under control of an IPTG-inducible promoter. In the present study, we modified our original protocol and incubated multiple cultures concurrently for a fixed period of time and then varied the timing of IPTG addition and the duration of IPTG induction for each culture. As shown in Fig. 1A, the abundance of Hap precursor and Hap_{β} in outer membranes and Hap_S in culture supernatants was detected by immunoblot analysis with an antiserum that reacts with Hap precursor, Hap_{β}, and Hap_S. Samples were normalized according to the quantity of major outer membrane protein P4 that was loaded. Based on analysis by scanning densitometry, the amount of Hap_{β} and Hap_S increased exponentially with increasing duration of IPTG induction (Fig. 1B), a finding consistent with previous results (4). In contrast, the amount of Hap precursor increased rapidly over the first 30 min of induction and then reached a plateau and remained almost unchanged between 30 and 60 min. These data suggest that after induction for 30 min, autoproteolysis had achieved maximum efficiency, capable of matching but not exceeding the rate of precursor production, resulting in a plateau in quantity of Hap precursor.

Interestingly, even though some Hap precursor was present in the uninduced culture due to leakiness of the *trc* promoter, Hap_S and Hap_{β} were hardly detectable in uninduced samples (Fig. 1A), indicating that autoproteolysis was minimal at low levels of Hap expression. With this observation in mind, we hypothesized that *H. influenzae* clinical isolates expressing Hap from a single chromosomal allele might demonstrate minimal Hap autoproteolysis. To further explore this possibility, we replaced the nonfunctional *hap* allele in *H. influenzae* strain Rd with either wild-type *hap* or a mutant gene that encodes HapS243A. Similar to observations with uninduced DB117/pJS106::Trc, immunoblot analysis of outer membranes and culture supernatants from Rd/wt-Hap revealed significant quantities of the 155-kDa Hap precursor, but relatively little Hap_S or Hap_{β}, even after prolonged film exposure times (Fig. 2). In outer membranes from Rd/HapS243A, we observed slightly more Hap precursor than in outer membranes from Rd/wt-Hap, confirming that wild-type Hap expressed from a chromosomal allele undergoes autoproteolysis, although only to a limited extent. In contrast, examination of DB117/pJS106, a *rec-1* derivative of Rd that expresses wild-type Hap from a multicopy plasmid, revealed equivalent amounts of Hap precursor and Hap_{β} in the outer membrane and abundant Hap_S in the culture supernatant, indicating that higher levels of Hap expression resulted in efficient autoproteolysis.

Taken together, these results suggest that a minimum threshold of Hap precursor expression is required for autoproteolysis and that levels of Hap associated with expression from a chromosomal allele are just above this threshold.

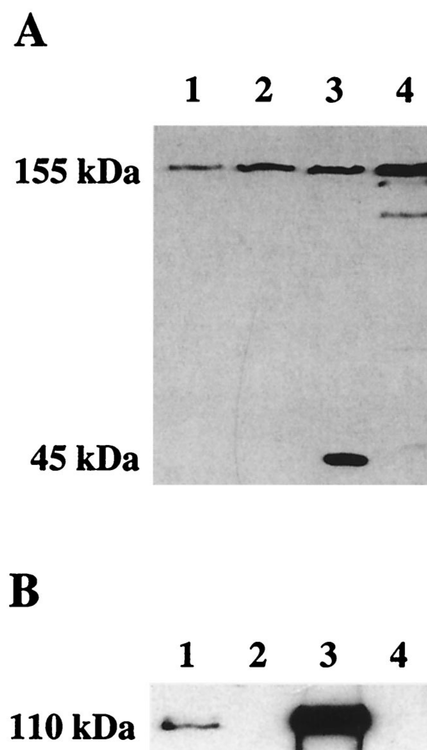

FIG. 2. Hap autoproteolysis in *H. influenzae* strains expressing Hap from a chromosomal allele or a multicopy plasmid. Outer membrane proteins (A) and culture supernatant proteins (B) from late-exponential-phase cultures of Rd/wt-Hap, Rd/HapS243A, DB117/pJS106, and DB117/pHapS243A are shown. Proteins were assessed by immunoblotting with antiserum Rab730, which reacts with Hap precursor, Hap_S, and Hap_{β}. The gels in both panels were loaded as follows: lane 1, Rd/wt-Hap; lane 2, Rd/HapS243A; lane 3, DB117/pJS106 (wild-type Hap); lane 4, DB117/pHapS243A.

A minimal quantity of Hap is sufficient for significant adherence. To address how the threshold for autoproteolysis may influence Hap function during *H. influenzae* infection, we examined our samples with various levels of Hap expression in adherence assays with Chang epithelial cells. Given that Hap-mediated adherence would be expected to depend on the amount of surface-exposed Hap_S, we initially analyzed intact whole bacteria from the IPTG induction experiment by dot immunoblot. Our goal was to quantify surface-exposed Hap_S. As shown in Fig. 3A, Hap_S was faintly detectable on the surface of uninduced DB117/pJS106::Trc, a finding consistent with the small amount of Hap precursor present in the outer membrane. Increasing induction with IPTG resulted in increasing Hap_S on the bacterial cell surface up to the 30-min time point, and then stable levels of Hap_S between 30 and 60 min. When these cultures were evaluated for binding to Chang cells, we observed a stepwise increase in adherence with increasing induction times (Fig. 4), correlating with the results obtained from immunoblots of outer membranes and intact whole bacteria. Adherence by uninduced bacteria expressing very low levels of Hap was increased threefold compared with

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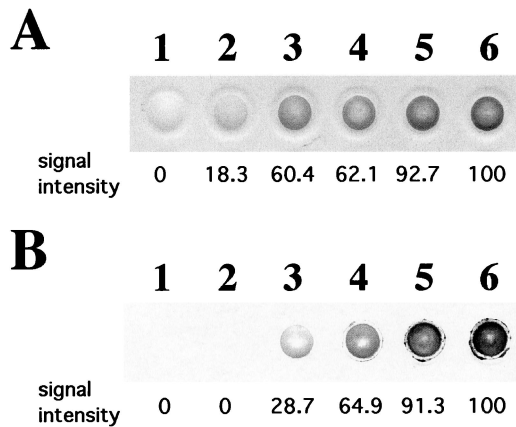

FIG. 3. Surface expression of Hap_S in *H. influenzae* strains expressing Hap from a chromosomal allele or a multicopy plasmid. (A) Dot immunoblot of intact whole bacteria of DB117 expressing wild-type Hap under control of an IPTG-inducible promoter. Samples were loaded as follows: well 1, DB117/pGJB103 (vector); wells 2 to 6, DB117/pJS106::Trc induced for 0, 10, 20, 30, and 60 min, respectively. (B) Dot immunoblot of intact whole bacteria of strains Rd or DB117 expressing wild-type Hap or HapS243A. Samples were loaded as follows: well 1, Rd; well 2, DB117/pGJB103 (vector); well 3, Rd/wt-Hap; well 4, Rd/HapS243A; well 5, DB117/pJS106 (wild-type Hap); well 6, DB117/pHapS243A. Immunoblot analysis was performed with antiserum GP74, which reacts with Hap_S. In both panels A and B, bacteria were fixed and washed before immunoblot analysis. The numbers under the wells represent relative signal intensity determined by using the NIH Image 1.62 program.

DB117 harboring vector alone ($P < 0.05$), emphasizing the capacity for Hap-mediated adherence even with minimal amounts of surface-associated Hap_S.

To extend these findings, we performed quantitative adherence assays comparing Rd expressing either wild-type Hap or HapS243A from a chromosomal allele and DB117 expressing either wild-type Hap or HapS243A from a multicopy plasmid. Dot immunoblot analysis revealed faintly detectable Hap_S on the surface of Rd/wt-Hap and increased Hap_S on the surface of Rd/HapS243A (Fig. 3B). Surface-exposed Hap_S was slightly more abundant in DB117/pJS106 than in Rd/HapS243A and was most abundant in DB117/pHapS243A (Fig. 3B). Adherence to Chang cells correlated well with Hap_S surface expression. Compared with adherence by Rd and DB117/pGJB103, adherence by Rd/wt-Hap was increased fivefold ($P < 0.05$), adherence by Rd/HapS243A and DB117/pJS106 was increased ~15-fold, and adherence by DB117/pHapS243A was increased ~35-fold (Fig. 5). Adherence assays with A549 cells and with extracellular matrix proteins fibronectin and collagen IV yielded similar results (Fig. 5). In all of these experiments, adherence by Rd/wt-Hap was 2- to 15-fold greater than background ($P < 0.05$). Adherence by Rd/wt-Hap was most striking in experiments with collagen IV.

Previous studies of Hap-mediated adherence demonstrated that incubation of DB117/pJS106 with SLPI, a serine protease inhibitor in human respiratory tract secretions that interferes with Hap autoproteolysis, resulted in increased adherence, approaching the levels observed with DB117/pHapS243A (7). To determine the effect of SLPI on strains expressing Hap from a chromosomal allele, we incubated Rd/wt-Hap with 8.5 μM SLPI prior to inoculation of Chang cells in a quantitative adherence assay. After pretreatment with SLPI, adherence by Rd/wt-Hap was comparable to adherence by Rd/HapS243A (Fig. 6), thus confirming our earlier results with multicopy Hap expression and further suggesting that *H. influenzae* clinical isolates might take advantage of SLPI in the respiratory tract to augment intimate association with host tissues. Adherence by Rd/HapS243A to Chang cells was not enhanced by pretreatment with SLPI, indicating that the effect of SLPI on adherence by Rd/wt-Hap was due solely to inhibition of Hap autoproteolysis.

Hap-mediated bacterial aggregation is proportional to the abundance of surface-exposed Hap_S above a minimum threshold. Beyond mediating adherence to epithelial cells and extracellular matrix proteins, Hap promotes bacterial aggregation and microcolony formation via interactions between Hap_S passenger domains on the surface of neighboring organisms. To extend our analysis of Hap-mediated adherence, we examined how levels of Hap expression might affect Hap-mediated bacterial aggregation and microcolony formation, as assessed by using a tube settling assay (Fig. 7A). Of note, tube settling provides a quantitative measure of bacterial aggregation and microcolony formation (D. L. Fink, A. Z. Buscher, B. Green, P. Fernsten, and J. W. St. Geme III, unpublished data). Examination of cultures from our IPTG induction experiment revealed that the culture of uninduced DB117/pJS106::Trc did not settle over 4 h, a result similar to that achieved with a culture of DB117 expressing empty vector. The lack of appreciable aggregation in the uninduced culture was somewhat surprising, given that the background level of Hap expression

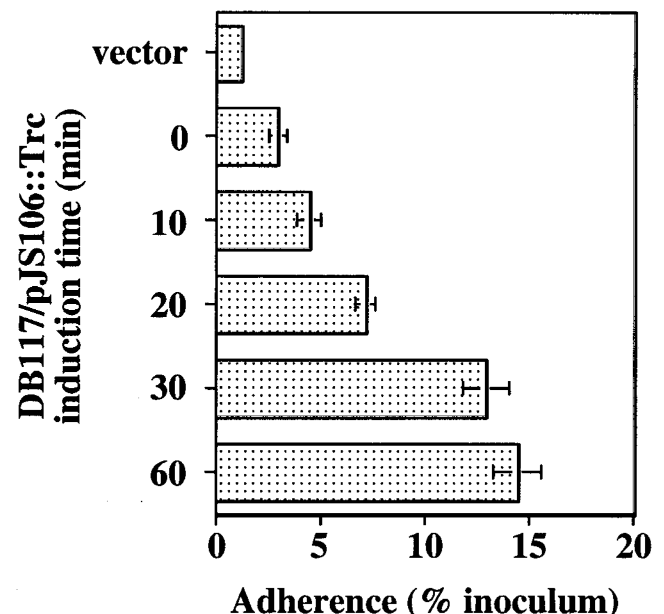

FIG. 4. Adherence to Chang cells by *H. influenzae* strain DB117 expressing Hap under control of an IPTG-inducible promoter. Cultures of DB117/pJS106::Trc or DB117/pGJB103 (vector) were grown to late exponential phase with induction by IPTG for the indicated durations. Adherence to Chang cells was calculated by dividing the number of adherent bacteria by the number of inoculated bacteria. Bars represent the means ± the standard error of the means of measurements made in triplicate from a representative experiment. In all cases, adherence by samples of DB117/pJS106::Trc was statistically significantly different from adherence by DB117/pGJB103 ($P < 0.05$).

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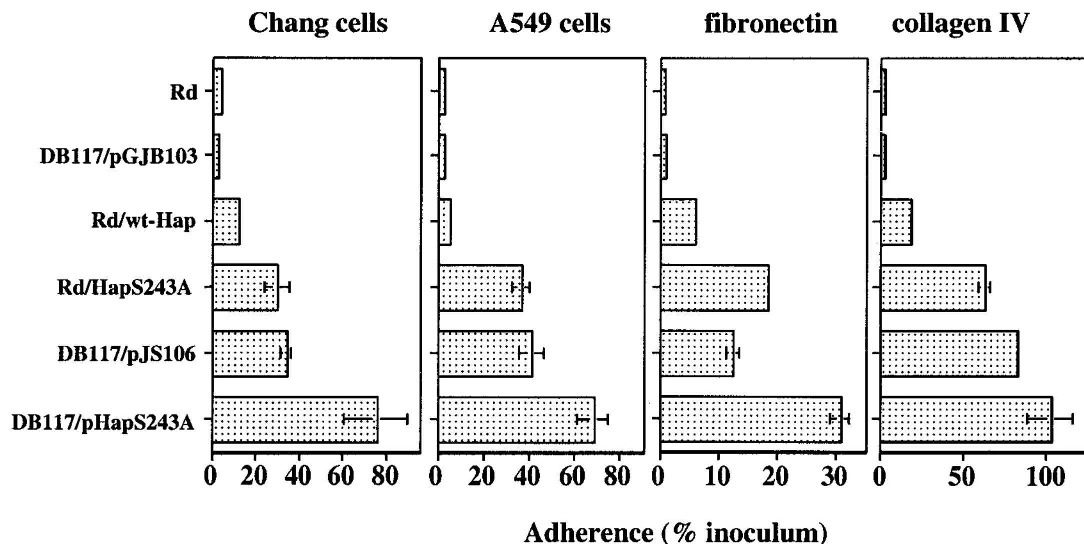

FIG. 5. Adherence to epithelial cell monolayers and extracellular matrix proteins by *H. influenzae* strains expressing wild-type Hap or HapS243A from a chromosomal allele or a multicopy plasmid. Adherence to Chang cells, A549 cells, fibronectin, and collagen IV was calculated by dividing the number of adherent bacteria by the number of inoculated bacteria. Bars represent the means ± the standard error of the means of measurements made in triplicate from representative experiments. Strains included Rd, DB117/pGJB103 (vector), Rd/wt-Hap, Rd/HapS243A, DB117/pJS106 (wild-type Hap), and DB117/pHapS243A. Adherence by Rd/wt-Hap, Rd/HapS243A, DB117/pJS106 (wild-type Hap), and DB117/pHapS243A was statistically significantly different from adherence by Rd and DB117/pGJB103 ($P < 0.05$).

in this strain was sufficient to promote adherence to Chang cells. In the remaining cultures, we observed a stepwise increase in settling rates with increasing induction times, a finding consistent with the levels of surface-associated Hap_S as measured by immunoblots of outer membranes and whole bacteria.

In additional experiments, we evaluated our strains expressing chromosomally or plasmid-encoded wild-type Hap or HapS243A in tube settling assays (Fig. 7B). DB117/pHapS243A exhibited the fastest settling rate, followed by DB117/pJS106 and Rd/HapS243A. Settling rates for these strains correlated well with expression levels of surface-associated Hap precursor, as indicated by dot immunoblot. Rd/wt-Hap demonstrated no appreciable settling over a 4-h assay, a finding reminiscent of the situation with uninduced DB117/pJS106::Trc and suggesting again that low-level Hap expression is insufficient to promote bacterial aggregation. Treatment of Rd/wt-Hap with 8.5 μM SLPI promoted appreciable aggregation by this strain, a finding similar to the aggregation promoted by Rd/HapS243A. Taken together, these data suggest that there is a minimum threshold of Hap expression required for bacterial aggregation and that single copy expression of Hap by *H. influenzae* clinical isolates may promote microcolony formation only in the context of impaired autoproteolysis, as might occur in the presence of SLPI.

DISCUSSION

In previous work, we established that the *H. influenzae* Hap autotransporter promotes adherence to cultured respiratory epithelial cells and selected extracellular matrix proteins and facilitates bacterial aggregation and microcolony formation (5, 7, 16). Hap adhesive activity is mediated by the surface-associated Hap_S passenger domain, which also has protease activity and directs autoproteolytic cleavage of itself from the membrane-associated Hap_β translocator domain (6). Hap autoproteolysis occurs via an intermolecular mechanism that depends on the density of Hap precursor on the bacterial cell surface (5). Inhibition of autoproteolysis by site-directed mutatgenesis

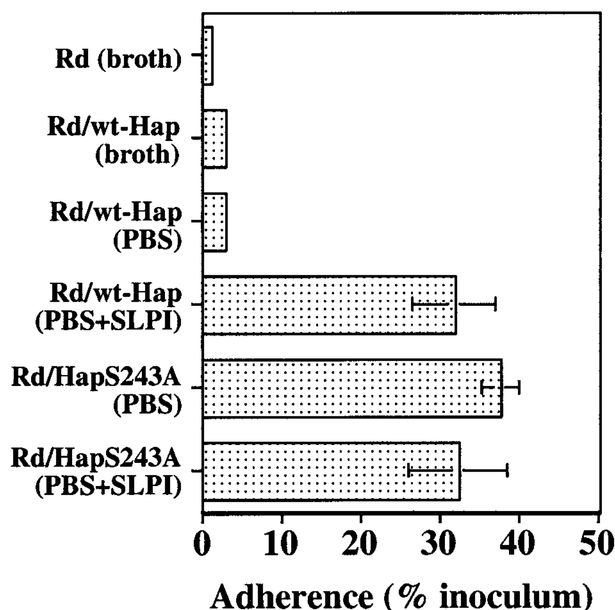

FIG. 6. Effect of SLPI on adherence to Chang cells by *H. influenzae* strain Rd/wt-Hap. Bacteria were inoculated onto monolayers either directly from broth cultures (no treatment) or after incubation in PBS with or without SLPI for 45 min at 37°C. Adherence to Chang cells was calculated by dividing the number of adherent bacteria by the number of inoculated bacteria. Bars represent the the means ± the standard errors of the means of measurements made in triplicate from a representative experiment. Adherence by Rd/wt-Hap and Rd/HapS243A, with or without SLPI, was statistically significantly different from adherence by Rd.

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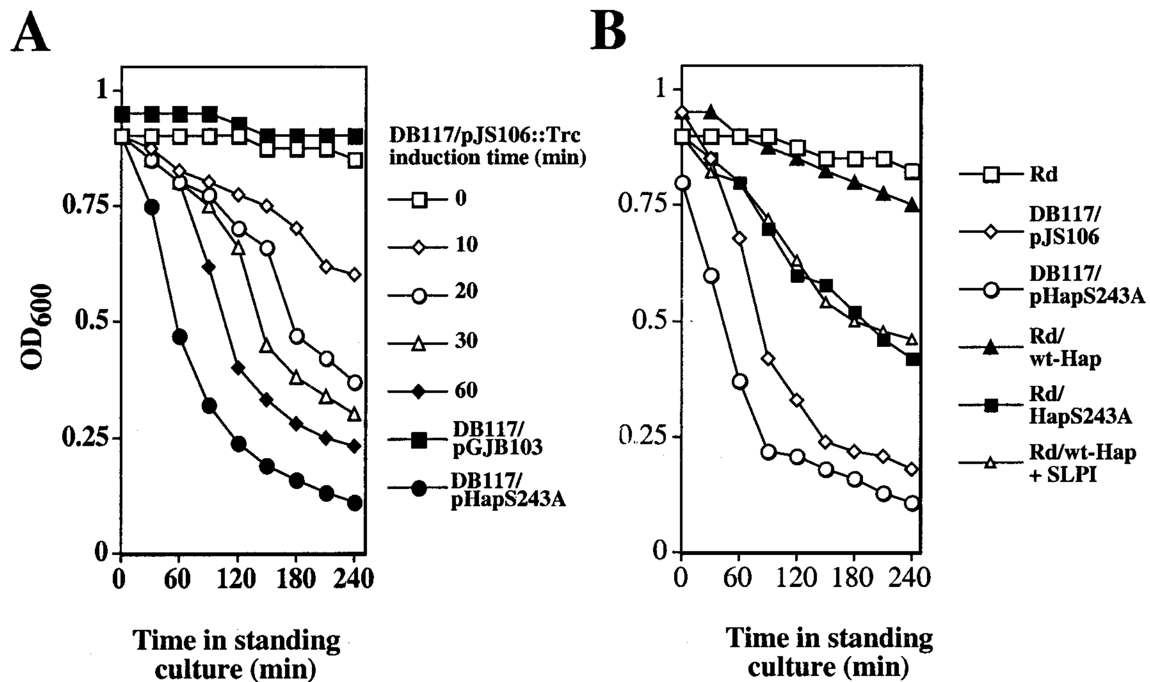

FIG. 7. Settling of cultures of *H. influenzae* strains expressing Hap from a chromosomal allele or a multicopy plasmid. (A) Late-exponential-phase cultures of DB117/pGJB103 (vector), DB117/pHapS243A, or DB117 expressing IPTG-induced wild-type Hap were incubated standing at room temperature for 4 h, and bacterial aggregation was quantitated by measuring the absorbance at 600 nm. (B) Cultures of DB117 or Rd expressing wild-type Hap or HapS243A were incubated standing at room temperature for 4 h, and bacterial aggregation was quantitated by measuring absorbance at 600 nm. In the sample labeled "Rd/wt-Hap + SLPI," Rd/wt-Hap was incubated in PBS plus 8.5 μM SLPI, washed once with PBS, and then resuspended in BHI.

of catalytic triad residues or treatment of bacteria with physiologic concentrations of SLPI results in retention of Hap_S on the bacterial cell surface and increased capacity for bacterial adherence and aggregation (7).

In the present study, we confirmed that the rate of Hap autoproteolysis increases exponentially with increasing expression of Hap precursor, a finding consistent with an intermolecular mechanism. At high levels of Hap expression, autoproteolysis is limited solely by the catalytic rate of peptide bond cleavage. When the density of Hap precursor on the bacterial cell surface is low, as with background expression in uninduced DB117/pJS106::Trc, autoproteolysis likely depends not only on the catalytic rate but also on factors that limit interaction between precursor molecules, such as membrane diffusion. Efficient autoproteolysis therefore requires a minimum threshold of precursor expression. Comparison of bacteria expressing Hap from a chromosomal allele and bacteria expressing Hap from a multicopy plasmid highlights the dependence of autoproteolysis on precursor density. In DB117/pJS106, Hap undergoes efficient autoproteolysis and Hap_S is abundant in the culture supernatant; in contrast, in Rd/wt-Hap, most Hap precursor remains uncleaved, indicating that Hap expression barely exceeds the threshold for autoproteolysis.

Limited secretion of Hap_S from the bacterial cell surface in the context of chromosomal expression suggests that autoproteolysis may not undermine the potential contribution of Hap adhesive activity to *H. influenzae* colonization. Rd/wt-Hap and uninduced DB117/pJS106::Trc adhered to epithelial cells two- to fivefold more efficiently than background (Rd and DB117/vector), indicating that Hap-mediated bacterial adherence is significant even when Hap expression is low. These results are consistent with previous studies examining the contribution of Hap to interactions between nontypeable *H. influenzae* clinical isolate N187 and Chang cells (7). Adherence of Rd/wt-Hap to fibronectin and collagen IV was even more impressive than adherence to epithelial cells, suggesting that Hap may serve primarily to facilitate *H. influenzae* adherence to extracellular matrix proteins. Along these lines, it is noteworthy that *H. influenzae* associates preferentially with damaged epithelium and exposed basement membrane in experiments with nasopharyngeal tissue in organ culture (12).

In studies with chromosomal expression of Hap, inhibition of Hap autoproteolysis by mutation of the active site serine significantly enhanced bacterial adherence to both epithelial cells and extracellular matrix proteins. Compared to Rd/wt-Hap, Rd/HapS243A adhered approximately threefold more efficiently to Chang cells, fibronectin, and collagen IV and approximately ninefold more efficiently to A549 cells. Based on quantification of surface-associated Hap_S in Rd/HapS243A and Rd/wt-Hap, it appears that modest increases in the abundance of Hap_S on the bacterial surface lead to substantial increases in Hap-mediated bacterial adherence. Thus, inhibition of autoproteolysis may serve to tightly regulate Hap adhesive activity in wild-type strains of *H. influenzae*. In the context of respiratory tract infection by clinical isolates, inhibition of autoproteolysis would most likely occur due to SLPI or other host protease inhibitors. Consistent with this hypothesis, pretreatment of Rd/wt-Hap with a physiologic concentration

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of SLPI increased adherence by this strain to levels comparable to adherence by Rd/HapS243A.

In contrast to the situation with adherence to epithelial cells and extracellular matrix proteins, Hap-mediated bacterial aggregation was not appreciable in settling assays with Rd/wt-Hap. Inhibition of Hap autoproteolysis by mutation of S243 or treatment with SLPI resulted in a marked increase in aggregation in tube settling assays, suggesting that the minimum threshold of surface-associated Hap_S required for aggregation exceeds the level associated with chromosomal expression of wild-type Hap. Thus, inhibition of autoproteolysis may add yet another layer of complexity to the regulation of Hap adhesive activities by influencing not only the capacity for adherence by single organisms but also the number of organisms associating with a particular site on the respiratory mucosal surface.

The observation that Rd/HapS243A and SLPI-treated Rd/wt-Hap are capable of forming bacterial aggregates raises the possibility that Hap may contribute to biofilm formation by *H. influenzae* clinical isolates. *H. influenzae* biofilms have been observed with experimentally infected animals and tympanostomy tubes from children with recurrent otitis media and may contribute to persistence in the face of antimicrobial therapy and the host immune response (3, 11). When conditions become more favorable for survival, organisms may disperse from biofilms and spread to new sites of infection. Decreases in local SLPI concentrations as inflammation subsides may permit Hap autoproteolysis, ultimately allowing release of bacteria from aggregates. As an alternative, *H. influenzae* may actively control the efficiency of Hap autoproteolysis by regulating expression levels of Hap precursor. It is possible that Hap expression by *H. influenzae* clinical isolates varies in response to changes in growth phase, bacterial density, or local environment.

In considering how Hap-mediated bacterial adherence and aggregation may contribute to *H. influenzae* pathogenesis, we speculate that autoproteolysis may serve to regulate Hap adhesive activities throughout progressive stages of natural infection. Hap autoproteolysis may occur to a limited extent during initial colonization of the respiratory tract by wild-type strains. In this setting, low-level Hap-mediated adherence to epithelial cells may complement the binding activity of other adhesins as individual organisms search for favorable sites of attachment along the mucosal surface. High-affinity interactions between Hap and extracellular matrix proteins may direct bacteria to damaged epithelium and exposed basement membrane in preparation for invasion into the subepithelium. Once bacteria trigger an immune response or migrate into areas of preexisting inflammation, inhibition of Hap autoproteolysis by SLPI, which is upregulated by tumor necrosis factor alpha and interleukin-1β (13), may facilitate persistence of *H. influenzae* by initiating biolfilm formation and strengthening existing interactions with epithelial cells and extracellular matrix.

In summary, the intermolecular mechanism of Hap autoproteolysis limits secretion of the adhesive Hap_S passenger domain in the context of low-level Hap expression, as occurs with *H. influenzae* clinical isolates. Chromosomal expression of wild-type Hap promotes low-level bacterial adherence to epithelial cells and moderate-level adherence to fibronectin and collagen IV but fails to mediate bacterial aggregation. With chromosomal expression of HapS243A, surface-associated Hap_S significantly enhances Hap-mediated adherence to epithelial cells and extracellular matrix proteins and exceeds the threshold required for Hap-mediated bacterial aggregation. Similar increases in adherence and aggregation occur with inhibition of Hap autoproteolysis by SLPI.

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