

Haemophilus ducreyi SapA Contributes to Cathelicidin Resistance and Virulence in Humans[∇]

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Haemophilus ducreyi is an extracellular pathogen of human epithelial surfaces that resists human antimicrobial peptides (APs). The organism's genome contains homologs of genes sensitive to antimicrobial peptides (*sap* operon) in nontypeable *Haemophilus influenzae*. In this study, we characterized the *sap*-containing loci of *H. ducreyi* 35000HP and demonstrated that *sapA* is expressed in broth cultures and *H. ducreyi*-infected tissue; *sapA* is also conserved among both class I and class II *H. ducreyi* strains. We constructed a nonpolar *sapA* mutant of *H. ducreyi* 35000HP, designated 35000HP*sapA*, and compared the percent survival of wild-type 35000HP and 35000HP*sapA* exposed to several human APs, including α -defensins, β -defensins, and the cathelicidin LL-37. Unlike an *H. influenzae sapA* mutant, strain 35000HP*sapA* was not more susceptible to defensins than strain 35000HP was. However, we observed a significant decrease in the survival of strain 35000HP*sapA* after exposure to LL-37, which was complemented by introducing *sapA* in *trans*. Thus, the Sap transporter plays a role in resistance of *H. ducreyi* to LL-37. We next compared mutant strain 35000HP*sapA* with strain 35000HP for their ability to cause disease in human volunteers. Although both strains caused papules to form at similar rates, the pustule formation rate at sites inoculated with 35000HP*sapA* was significantly lower than that of sites inoculated with 35000HP (33.3% versus 66.7%; $P = 0.007$). Together, these data establish that SapA acts as a virulence factor and as one mechanism for *H. ducreyi* to resist killing by antimicrobial peptides. To our knowledge, this is the first demonstration that an antimicrobial peptide resistance mechanism contributes to bacterial virulence in humans.

Haemophilus ducreyi is the causative agent of chancroid, a genital ulcer disease that facilitates both the transmission and acquisition of HIV-1 (4, 45). An obligate human pathogen, *H. ducreyi* infects epithelial surfaces and primarily remains localized in the skin (49). During experimental human infection, polymorphonuclear leukocytes (PMNs) and macrophages are rapidly recruited to the bacterial entry site. The organism colocalizes with PMNs and macrophages throughout disease but remains extracellular (8, 10).

As part of the innate immune system's response to infection, PMNs, macrophages, and epithelial cells secrete antimicrobial peptides (APs). Most APs are small, cationic peptides that are secreted into the extracellular milieu and have both bactericidal and chemotactic properties (26). Cationic APs are attracted to the anionic bacterial cell membrane where they lyse the bacterial cell. Some APs specifically recruit macrophages, PMNs, T cells, and immature dendritic cells to the site of bacterial infection, serving as a bridge between innate and adaptive immunity (39, 40, 53).

In humans, the best-studied APs include the family of defensins, which are subdivided by structure into α - and β -defensins, and one human cathelicidin, LL-37. Defensins are cysteine- and arginine-rich β -sheet peptides that range from 29

to 47 amino acids in length (29). Defensins form three intramolecular disulfide bonds via six invariant cysteine residues. Both the positions of the cysteines and the pattern of disulfide bonding differ between the α - and β -defensins (17, 29). In contrast, cathelicidin LL-37 is devoid of cysteines and forms an α -helix (20).

H. ducreyi encounters several cellular sources of APs during human infection: keratinocytes constitutively express human β -defensin 1 (HBD-1) and upregulate expression of HBD-2, HBD-3, and LL-37 in response to inflammation (22, 23, 30, 54); PMNs secrete preformed α -defensins, including human neutrophil peptide 1 (HNP-1), HNP-2, HNP-3, and HNP-4, as well as HBD-4 and LL-37 during infection (16, 50); and macrophages secrete HBD-1, HBD-2, and LL-37 in response to inflammatory mediators (15, 31). Vaginal epithelial cells also constitutively express the α -defensin human defensin 5 (HD-5) (43). Dual staining for *H. ducreyi* and HNP-1, HNP-2, and HNP-3 demonstrate that *H. ducreyi* is exposed to APs at the papular, pustular, and ulcerative stages of disease (10; C. A. Townsend and M. E. Bauer, unpublished data). *H. ducreyi* resists the bactericidal effects of several human APs, including those predicted to be at the site of infection; this resistance has been observed in representative members of two phenotypic *H. ducreyi* classes (38, 51). The mechanism(s) by which *H. ducreyi* evades AP-mediated killing is unknown.

Bacterial pathogens have evolved many mechanisms to resist killing by APs, including enzymatic inactivation of APs, electrostatic repulsion by the addition of positively charged resi-

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TABLE 1. Bacterial strains and plasmids used in this study

| Strain or plasmid | Genotype or description and origin | Source or reference(s) |
|---------------------------|--|------------------------|
| <i>E. coli</i> strains | | |
| HB101 | F <i>thi-1 hsdS20</i> (r _B m _B) <i>supE44 recA13 ara-14 leuB6 proA2 lacY1 galK2 rpsL20 (strR) xyl-5 mtl-1</i> | Promega |
| TOP10 | F ⁻ <i>mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araΔ139 Δ(ara-leu)7697 galU galK rpsL (strR) endA1 nupG</i> | Invitrogen |
| <i>H. ducreyi</i> strains | | |
| 35000HP | Class I clinical isolate; human-passaged variant of strain 35000, Winnipeg, Canada, 1975 | 2, 21, 51 |
| HD183 | Class I clinical isolate; Singapore, 1982 | 9, 48 |
| HD188 | Class I clinical isolate; Kenya, 1982 | 9, 48 |
| 82-029362 | Class I clinical isolate; California, 1982 | 9, 48 |
| 6644 | Class I clinical isolate; Massachusetts, 1989 | 9, 48 |
| HD85-023233 | Class I clinical isolate; New York, 1985 | 9, 48 |
| CIP542-ATCC | Class II clinical isolate; Hanoi, Viet Nam, 1954 | 21, 51 |
| HMC112 | Class II clinical isolate; 1984 (location not reported) | 51 |
| 33921 | Class II clinical isolate; Kenya (yr not reported) | 9, 13 |
| DMC164 | Class II clinical isolate; Bangladesh (yr not reported) | 51 |
| MEB107 | 35000HP <i>sapA::kan</i> | This study |
| MEB121 | 35000HP <i>sapA::kan/psapA</i> | This study |
| Plasmids | | |
| pGEM-T Easy | TA cloning vector; Amp ^r ^a | Promega |
| pSC-A | TA cloning vector; Amp ^r | Stratagene |
| pUC18K2 | Source of nonpolar Kan ^r cassette | 37 |
| pRSM2072 | <i>H. ducreyi</i> suicide vector | 12 |
| pLSSK | <i>H. ducreyi</i> shuttle vector | 52 |
| pMEB078 | 5' end of <i>sapA</i> in pGEM-T Easy | This study |
| pMEB080 | 3' end of <i>sapA</i> in pGEM-T Easy | This study |
| pMEB092 | 5' end of <i>sapA</i> + Kan ^r cassette in pGEM-T Easy | This study |
| pMEB098 | 5' end of <i>sapA</i> + Kan ^r cassette + 3' end of <i>sapA</i> in pGEM-T Easy | This study |
| pMEB108 | <i>sapA</i> ORF in pGEM-T-Easy | This study |
| pMEB114 | Putative <i>sap</i> promoter in pSC-A | This study |
| pMEB115 | Putative <i>sap</i> promoter + <i>sapA</i> ORF in pGEM-T Easy | This study |
| pMEB120 | Putative <i>sap</i> promoter + <i>sapA</i> ORF in pLSSK | This study |

^a Amp^r denotes resistance to ampicillin.

dues on the surface, and expression of transporters that remove APs before they can attack the cell membrane (27). To identify putative AP resistance factors in *H. ducreyi*, we examined the *H. ducreyi* genome (<http://stdgen.northwestern.edu>) for evidence of homology with previously described resistance strategies in other bacteria. Using a BLASTP search, we found that the *H. ducreyi* genome included homologs of the previously described transporter genes sensitive to antimicrobial peptides (*sap* genes) (3). The *sap* genes encode the Sap influx pump, which confers resistance to APs in several Gram-negative pathogens, including *Salmonella enterica* serovar Typhimurium, nontypeable *Haemophilus influenzae*, *Proteus mirabilis*, and *Erwinia chrysanthemi* (32, 35, 36, 41).

The Sap transporter consists of five proteins: SapB and SapC are permease proteins that form a pore in the bacterial inner membrane, SapD and SapF function as ATPase subunits of the transporter, and SapA is a periplasmic solute binding protein (34, 41). The nontypeable *H. influenzae* SapA protein has been shown to directly bind APs, including both LL-37 and HBD-3 (34). Once an AP enters the periplasm, SapA is thought to shuttle the AP to the SapBCDF channel, where the AP is transported into the cytoplasm, bypassing direct interaction between the AP and the cytoplasmic membrane, which would be the lethal event in AP attack (34, 41). Once in the cytoplasm, the fate of the AP is unknown, but presumably, it is degraded and its amino acids are recycled.

Sap transporters take up a variety of APs; among human APs tested, the *S. enterica* serovar Typhimurium Sap transporter confers resistance to crude extracts of human PMNs, and the nontypeable *H. influenzae* Sap transporter confers resistance to LL-37 and HBD-3 (19, 34, 35). Further, the nontypeable *H. influenzae* SapA protein has been shown to directly bind APs, including both LL-37 and HBD-3 (34).

Because Sap transporters protect other pathogens from APs, we hypothesized that the putative *H. ducreyi* Sap transporter system would confer resistance against APs and that the loss of the periplasmic component SapA would result in increased susceptibility to APs *in vitro* and loss of virulence *in vivo*. Herein, we describe the construction of an isogenic *sapA* insertion-deletion mutant to test this hypothesis and to define a mechanism for AP resistance of *H. ducreyi*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. *H. ducreyi* strains were grown on chocolate agar plates at 33°C with 5% CO₂. Liquid cultures were grown with aeration at 33°C in gonococcal broth (15% proteose peptone [BD, Sparks, MD], 4% K₂HPO₄, 2% KH₂PO₄, 10% NaCl) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 1% IsoVitalX, and 50 μg of hemin (Aldrich Chemical Co., Milwaukee, WI) per ml (5). *Escherichia coli* strains were grown in Luria-Bertani (LB) broth at 37°C with aeration or on LB plates at 37°C (44).

Nested RT-PCR. RNA was isolated from mid-logarithmic cultures of *H. ducreyi* 35000HP and from a biopsy tissue specimen of *H. ducreyi*-infected skin

TABLE 2. Primers used in this study

| Primer | Construct or use(s) | Sequence ^a |
|-----------------|---|---|
| 5'SapA Forward | pMEB078 | CATATC <u>tctaga</u> GCGGATAGCTTAATTTATTGCACCAG |
| 5'SapA Reverse | pMEB078 | CATATC <u>ggtacc</u> CATATC <u>cctcgag</u> GAATCTTTACTGTATA TTCATTCGGTGCTG |
| 3'SapA Forward | pMEB080 | CATATC <u>tctaga</u> GCGGATAGCTTAATTTATTGCACCAG |
| 3'SapA Reverse | pMEB080 | TAAACGAACCTTGCCCGAATGGCTC |
| SapAcompFor1 | pMEB108 | GATGTATCATTACTACTAATATCCCTGCT |
| SapAcompRev1 | pMEB108 | CGGTCAAATCCAACAGAACACAG |
| SapPromoterFor1 | pMEB114 | CCTTTAATTTGTTCTAAATACATAATGATCC |
| SapPromoterRev1 | pMEB114 | CATATC <u>cctatg</u> TCTTTTCGCCTTAATTTAAGC |
| Sap Forward 2 | Nested RT-PCR | CGGATAATTCGGTGTGGCGCATT |
| Sap Reverse 3 | Nested RT-PCR | TGTGCACTCGACCTGTATGCGGAT |
| Sap Forward 5 | PCR of clinical isolates | GCATTTCTCATACTGCGGTGGCAA |
| Sap Forward 6 | Nested RT-PCR | AATGCGCATATTGACGGTGGGG |
| Sap Reverse 6 | Nested RT-PCR; PCR of clinical isolates | ATCCGAGCGGAATCCCGATAATCA |

^a Restriction enzyme sites are shown in lowercase type and underlined.

obtained from a volunteer, using the Ultraspec reagent (Clontech, Mountainview, CA) as previously described (7). cDNA was synthesized using random hexamers (Clontech). Reverse transcriptase PCR (RT-PCR) was performed using HiFi *Taq* (Invitrogen, Carlsbad, CA) and the Sap Forward 2 and Sap Reverse 3 primers (Table 2). An annealing temperature of 60°C was used for 45 cycles of PCR amplification to produce a 569-bp product. Nested RT-PCR was performed using 2 µl of the previous reaction mixture as a template and the Sap Forward 6 and Sap Reverse 6 primers to amplify a 194-bp nested SapA RT-PCR product. Samples lacking template and samples lacking RT were included in all reaction mixtures.

Survey of clinical isolates of *H. ducreyi* for *sapA* gene. Chromosomal DNA was isolated from six class I clinical *H. ducreyi* isolates and four class II clinical *H. ducreyi* isolates (Table 1), as described previously (9). A 2,032-bp fragment, including *sapA*, was PCR amplified using primers Sap Forward 5 and Sap Reverse 6 (Table 2). A reaction mixture containing no template served as a negative control. Amplimers were used as templates for sequencing the *sapA* open reading frames (ORFs), performed by the DNA Sequencing Core Facility at the Indiana University School of Medicine. Sequences were aligned using the ClustalW algorithm in the Lasergene software package (DNASTAR, version 7).

Construction and complementation of a *sapA* mutant. The primers used to generate constructs in this study are listed in Table 2. All plasmids were maintained in *E. coli* TOP10, and mutagenic and complementing plasmids were passed through *E. coli* HB101 before introduction into *H. ducreyi* (6). A 517-bp fragment containing the 5' end of HD1230 (*sapA*) was PCR amplified from *H. ducreyi* 35000HP genomic DNA. The amplicon was TA cloned into pGEM-T Easy (Promega, Madison, WI) to generate pMEB078. A 489-bp fragment containing the 3' end of HD1230 was PCR amplified from genomic DNA and TA cloned into pGEM-T Easy (Promega) to generate pMEB080. A nonpolar kanamycin resistance (Kan^r) cassette from pUC18K2 (37) was inserted downstream of the 5-prime fragment of *sapA* in *H. ducreyi* MEB078 to generate pMEB092. The *sapA'*-Kan^r fragment of pMEB092 was subcloned upstream of the 3-prime region of *sapA* in pMEB080, generating pMEB098. In the resulting mutagenic construct, 676 bp of *sapA* was replaced with the Kan^r cassette. The *sapA'*-Kan^r-*sapA* fragment was subcloned into pRSM2072, which expresses *lacZ* and acts as a suicide vector in *H. ducreyi* (12), and introduced into strain 35000HP by electroporation. Kanamycin-resistant transformants were propagated on chocolate agar plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) in order to select for colonies in which the wild-type allele had been successfully replaced by the mutagenized allele (12). The *sapA* mutation was confirmed by digestion, PCR analysis, Southern blotting, and DNA sequencing (data not shown). *H. ducreyi* 35000HP and 35000HP*sapA* demonstrated similar growth rates in broth (data not shown). Quantitative RT-PCR (qRT-PCR) was used to verify that transcription of downstream genes was unaffected by the *sapA* mutation (data not shown).

To complement *H. ducreyi* 35000HP*sapA* in *trans*, we expressed *sapA* under the control of its native promoter in the *H. ducreyi* shuttle vector pLSSK (52). The *sapA* ORF was PCR amplified and TA cloned into pGEM-T Easy (Promega) to generate pMEB108. The 5' primer used to amplify *sapA* inserted CAT bases immediately upstream of the *sapA* start codon to introduce an NdeI site (CAT ATG) at the 5' end of the *sapA* clone. Using BPROM software (SoftBerry, Inc., Mount Kisco, NY), a putative promoter was identified starting 177 bp upstream

of *tyrR* in an untranslated region. To place the putative promoter upstream of the *sapA* gene, sequence 237 bp upstream of *tyrR* was PCR amplified with primers that introduced an NdeI site at the 3' end and TA cloned into pSC-A (Stratagene, Cedar Creek, TX) to generate pMEB114. The putative promoter region was excised from pMEB114, digested with NdeI, and ligated with NdeI-digested pMEB108 to generate pMEB115, in which the *sapA* ORF lies immediately downstream of the untranslated region upstream of *tyrR*. This construct was subcloned into pLSSK on a NotI fragment to produce pMEB120, which was introduced into mutant strain 35000HP*sapA* by electroporation. Transformants were selected on plates containing kanamycin and streptomycin; one transformant was selected and designated 35000HP*sapA/psapA*. RT-PCR was used to verify that the *sapA* gene was transcribed in mid-logarithmic cultures of strain 35000HP*sapA/psapA* (data not shown).

Sources of peptides. Recombinant α- and β-defensins were purchased from PeptoTech Inc. (Rocky Hill, NJ), Sigma-Aldrich (St. Louis, MO), Peptides International (Louisville, KY), and AnaSpec (San Jose, CA). Synthetic LL-37 was purchased from Phoenix Pharmaceuticals, Inc. (Belmont, CA).

96-Well AP bactericidal assay. The 96-well AP bactericidal assay was performed as previously described (38). Briefly, bacteria were grown to mid-logarithmic phase and suspended in 10 mM sodium phosphate (pH 7.4) supplemented with 0.1% brain heart infusion broth (BD). Approximately 10³ CFU of bacteria were mixed in duplicate with the indicated concentration of peptides in wells of a 96-well polypropylene plate (catalog no. 3790; Costar) and incubated for 1 h at 33°C. The concentration of bacteria remaining in the wells was determined by counting three replicate plates, and the results from duplicate wells were averaged. Survival in the presence of APs was calculated as a percentage of survival in control wells without APs. Because we have observed lot-to-lot variations in the level of AP activity, comparisons among bacterial strains for AP susceptibility were made only when the strains were assayed using the same lot of AP.

Assessment of virulence of the *sapA* mutant in human volunteers. Human challenge studies with *H. ducreyi* were performed under the guidelines of the U.S. Department of Health and Human Services, the U.S. Food and Drug Administration, and the Institutional Review Board of Indiana University-Purdue University at Indianapolis. After giving informed consent for participation and HIV serology, six healthy, adult volunteers (4 males and 2 females; 3 white, 3 black; age range, 25 to 57 years; mean age ± standard deviation [SD], 44 ± 10 years) were inoculated on the upper arm with *H. ducreyi* 35000HP and 35000HP*sapA* and monitored to the clinical endpoint, exactly as described previously for parent-mutant trials in this model (1, 5, 24). Parameters compared between parent-inoculated and mutant-inoculated sites included rates of papule and pustule formation and day 1 lesion size.

There are gender and other host effects on susceptibility to disease progression in the human model (11, 46); subjects are inoculated with the parent and mutant strains and serve as their own controls for these effects. To account for within-subject correlation among inoculated sites, statistical analysis of papule and pustule formation rates utilized a logistic regression model with generalized estimating equations (GEE), as described previously (47). Ninety-five percent confidence intervals (95% CIs) were calculated using the GEE sandwich estimate for standard errors. Differences in the rates of papule or pustule formation between the two strains tested were considered statistically significant if *P* was

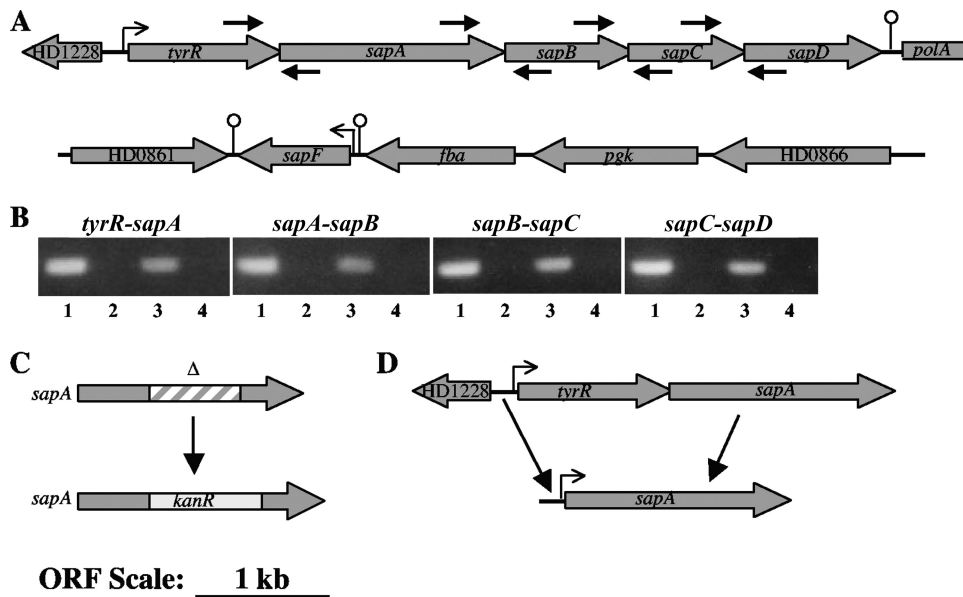


FIG. 1. Analysis and mutagenesis of the *sap* genes in *H. ducreyi* 35000HP. (A) Genetic map of *sap*-containing loci. An operon containing *tyrR* and *sapABCD* was located between ORFs HD1228 and HD1236, and the unlinked *sapF*-containing locus was located between ORFs HD0861 and HD0866. Arrowheads show the direction of transcription, stalked arrows represent predicted promoters, and ball-and-stick structures indicate predicted stem-loop transcriptional terminators. Primer binding sites are indicated by small arrows. (B) Operon mapping of the *sapABCD* locus. RT-PCR was used to amplify mRNA across junctions of the indicated adjacent genes. Lanes 1, chromosomal DNA; lanes 2, no-template control; lanes 3, RNA samples with RT; lanes 4, RNA samples with no RT added. (C) Insertion/deletion mutagenesis of *sapA*. A 676-bp fragment of *sapA* (hatched box) was replaced with a 840-bp nonpolar *Kan^r* cassette (gray box). This construct was inserted into the chromosome by allelic replacement as described in Materials and Methods. (D) Expression of *sapA* downstream of its native promoter for trans-complementation. *sapA* was cloned downstream of 237 bp of the untranslated sequence 5' of *tyrR* for expression from the native promoter. The cloned 5' sequence contained a predicted -35 and -10 region and ribosome binding site. Panels A, C, and D are drawn at the same scale.

<0.05. Statistical analysis of day 1 papule sizes was calculated using a two-tailed Student's *t* test.

RESULTS

Characterization of *sap* genes in *H. ducreyi*. Homology searches showed that *H. ducreyi* 35000HP contains genes whose predicted products were highly homologous to five of the nontypeable *H. influenzae* 86-028NP Sap proteins (3), including SapA (44% identity; 67% similarity), SapB (46% identity; 67% similarity), SapC (52% identity; 73% similarity), SapD (65% identity; 82% similarity), and SapF (59% identity; 73% similarity). Nontypeable *H. influenzae* also contains a sixth Sap protein, SapZ, which is found in some but not all bacteria containing Sap transporters; the function of SapZ is unknown (35). We found no homolog of SapZ in *H. ducreyi* 35000HP.

The locus containing *sapA* in *H. ducreyi* 35000HP is shown in Fig. 1A. A putative regulatory gene, *tyrR*, and a predicted promoter region were identified upstream of *sapA*, and homologs of *sapBCD* were identified downstream of *sapA*. A rho-independent terminator was predicted in the intergenic space between *sapD* and the next downstream gene, *polA* (55). Surprisingly, the *sapA* locus did not contain a *sapF* homolog; rather, the genome contained an unlinked *sapF* homolog that was flanked by predicted promoter and terminator regions, suggesting that *sapF* may be in a monocistronic operon (Fig. 1A). We used RT-PCR to map the operon structure of the *H. ducreyi sapABCD* locus and found that the four contiguous *sap*

genes, along with the upstream *tyrR*, are cotranscribed in a single operon (Fig. 1B).

The *H. ducreyi* Sap transporter genes are transcribed during human infection. We recently completed a study using selective capture of transcribed sequences (SCOTS) to identify genes important for *H. ducreyi* virulence (7). With SCOTS, the competitive hybridization of tissue-derived and broth-derived sequences is used to identify genes that may be preferentially expressed *in vivo* (18). Among the *in vivo*-derived genes identified were homologs of *H. influenzae sapA*, *sapB*, and *sapF* (7). To confirm *in vivo* expression of *sapA*, we performed nested RT-PCR on RNA from an *H. ducreyi*-infected pustule of a human volunteer. Transcripts were amplified in both tissue-derived RNA and broth-derived RNA (Fig. 2A). These data confirm that *H. ducreyi* expresses *sapA* at the pustular stage of disease during experimental human *H. ducreyi* infection.

Identification of *sap* genes by SCOTS suggested that *H. ducreyi* may upregulate *sap* genes *in vivo*. *H. ducreyi* encodes one two-component regulator, a homolog of CpxRA (28). Although we identified a putative CpxR recognition motif upstream of *tyrR*, qRT-PCR analysis showed no difference in *sapA* expression between isogenic *cpxR* or *cpxA* mutants and their parent strain 35000HP (data not shown) (14, 28). We also used qRT-PCR to examine whether exposure to cathelicidin LL37 (0.06 or 1 μ g/ml) signals *H. ducreyi* to upregulate *sapA*. Neither LL37 dose affected the level of *sapA* transcripts in strain 35000HP (data not shown).

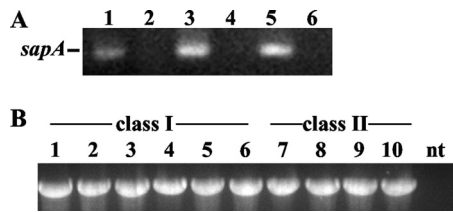


FIG. 2. *sapA* is expressed *in vivo* and conserved among *H. ducreyi* strains. (A) Nested RT-PCR of *sapA* transcripts was performed on RNA from broth culture (lanes 3 and 4) or a pustule biopsy specimen from the human model (lanes 5 and 6). Controls were included in which no RT was added to reaction mixtures (lanes 4 and 6) or no template was used (lane 2). Genomic DNA (lane 1) served as a positive control. (B) PCR amplification of *sapA*-containing sequence from class I and class II clinical isolates of *H. ducreyi* (see Table 1). A 2.032-kbp fragment was amplified from genomic DNA of each strain using primers flanking the *sapA* ORF in *H. ducreyi* 35000HP. Lanes 1 to 6 contain class I strains as follows: lane 1, 35000HP; 2, HD183; 3, HD188; 4, 82-029362; 5, 6644; 6, HD85-023233. Lanes 7 to 10 contain class II strains as follows: lane 7, CIP542-ATCC; 8, HMC112; 9, 33921; 10, DMC64. nt, no-template control.

The *sapA* gene is conserved among both class I and class II strains of *H. ducreyi*. Two phenotypic classes of *H. ducreyi* have been described (51). Our previous studies demonstrated that both classes of *H. ducreyi* are resistant to human APs (38). We examined whether a panel of 10 clinical isolates harbored the *sapA* gene. Using primers flanking the *sapA* ORF from the *H. ducreyi* 35000HP sequence, we PCR amplified genomic DNAs of all clinical isolates tested; amplicons comigrating with the amplicon from strain 35000HP were identified (Fig. 2B). Sequencing confirmed the conservation of the full-length, 1,683-bp *sapA* ORF in all 10 isolates analyzed. In three class I strains, the *sapA* sequence was identical to *sapA* in the prototype class I strain 35000HP. However, in class I strains 6644 and 82-029362, we found a single G-to-A base pair change, which predicted an amino acid change from R to H at position 132 of the protein sequence. The *sapA* genes in four class II strains were identical to each other and differed at 12 bp from the 35000HP *sapA* ORF; these substitutions resulted in 4 amino acid changes in the predicted protein sequence, including S to A at position 26, A to V at position 183, V to A at position 428, and Y to D at position 510. The class II strains did not contain the single base pair change detected in two of the class I strains. Overall, the sequence data show that *sapA* is highly conserved in both classes of *H. ducreyi*, including strains from several geographic locations.

Construction and complementation of a *sapA* mutant in *H. ducreyi*. To determine the role of SapA in AP resistance of *H. ducreyi*, we mutagenized *sapA* in *H. ducreyi* 35000HP by insertion of a nonpolar cassette (Fig. 1C). qRT-PCR showed that the transcription level of *sapD*, the most downstream gene within the operon, was unaffected by the mutagenesis procedure, confirming that the mutation in strain 35000HP*sapA* was nonpolar (data not shown). To confirm that phenotypic differences between strains 35000HP and 35000HP*sapA* were due solely to the loss of a functional *sapA* gene in the mutant, we cloned *sapA* downstream of its predicted native promoter sequence located upstream of *tyrR* (Fig. 1D) to *trans*-complement 35000HP*sapA*; the resulting strain was designated 35000HP*sapA*/psapA.

The *H. ducreyi sapA* gene confers resistance to the human cathelicidin LL-37. *H. ducreyi* is likely exposed to multiple sources of LL-37 during infection, and the bacterium is resistant to the bactericidal effects of this peptide (38). In a 96-well AP bactericidal assay with 10-fold serial dilutions of LL-37, *H. ducreyi* 35000HP*sapA* was significantly more sensitive than *H. ducreyi* 35000HP to LL-37 at all but the lowest concentration (Fig. 3A). Assays with 2-fold serial dilutions of LL-37 were performed to more specifically characterize the effect of the *sapA* mutation on *H. ducreyi* resistance to LL-37. Wild-type *H. ducreyi* demonstrated significantly greater levels of survival than the *sapA* mutant when challenged with 0.25 μ g/ml to 2 μ g/ml LL-37 (Fig. 3B). *trans*-Complementation with *sapA* expressed on pLSSK restored resistance to LL-37 (Fig. 3). Additional experiments showed that there were no differences in susceptibility of strains 35000HP*sapA* and 35000HP*sapA* transformed with vector pLSSK to LL-37 resistance (data not shown). These data suggest that the Sap transporter plays a role in protecting *H. ducreyi* from the bactericidal activity of LL-37.

The *H. ducreyi sapA* gene does not confer resistance to human defensins. *H. ducreyi* is exposed to PMN-derived α -defensins at all stages of disease (10; data not shown), and *H. ducreyi* is likely exposed to the α -defensin HD-5 during vaginal and cervical infection (43). Similarly, *H. ducreyi* is exposed to multiple cellular sources of β -defensins at the site of infection. *H. ducreyi* is resistant to the bactericidal activity of these human α - and β -defensins (38), and the Sap transporter has been shown to contribute to β -defensin resistance in other bacteria. Thus, we examined the role of the Sap transporter in *H. ducreyi* resistance to these human defensins. In a 96-well AP bactericidal assay with 10-fold serial dilutions of peptide, there was no discernible difference in AP resistance levels of *H. ducreyi* 35000HP and 35000HP*sapA* exposed to the α -defensins HNP-1, HNP-2, and HD-5 (Fig. 3C and data not shown). Similarly, we found no significant difference in the rate of survival of wild-type *H. ducreyi* and the 35000HP*sapA* mutant after exposure to increasing concentrations of the β -defensins HBD-2, HBD-3, and HBD-4 (Fig. 3D and data not shown). These findings indicate that the *H. ducreyi* Sap transporter is not required for the bacterium's resistance to the α -defensins or β -defensins tested.

The *H. ducreyi sapA* mutant is partially attenuated for virulence in humans. To determine whether SapA plays a role in disease, we tested *H. ducreyi* 35000HP*sapA* alongside 35000HP for virulence in the human model of *H. ducreyi* infection. Six healthy adults were challenged with *H. ducreyi* in two iterations (Table 3). In the first iteration, 3 volunteers were each inoculated with an estimated delivered dose of 81 CFU of *H. ducreyi* 35000HP at three sites on one arm and with estimated delivered doses of 42, 84, and 165 CFU of *H. ducreyi* 35000HP*sapA* at three sites on the other arm. Papules formed at all sites, and pustules formed at 6 of 9 parent-inoculated sites and at 5 of 9 mutant-inoculated sites (Table 3). In the second iteration, 3 volunteers received an estimated delivered dose of 97 CFU of strain 35000HP at each of three sites and estimated delivered doses of 47, 94, and 188 CFU of strain 35000HP*sapA* at three sites. Papules formed at all parent-inoculated sites and at 8 of 9 mutant-inoculated sites. Pustules formed at 6 of 9 parent-

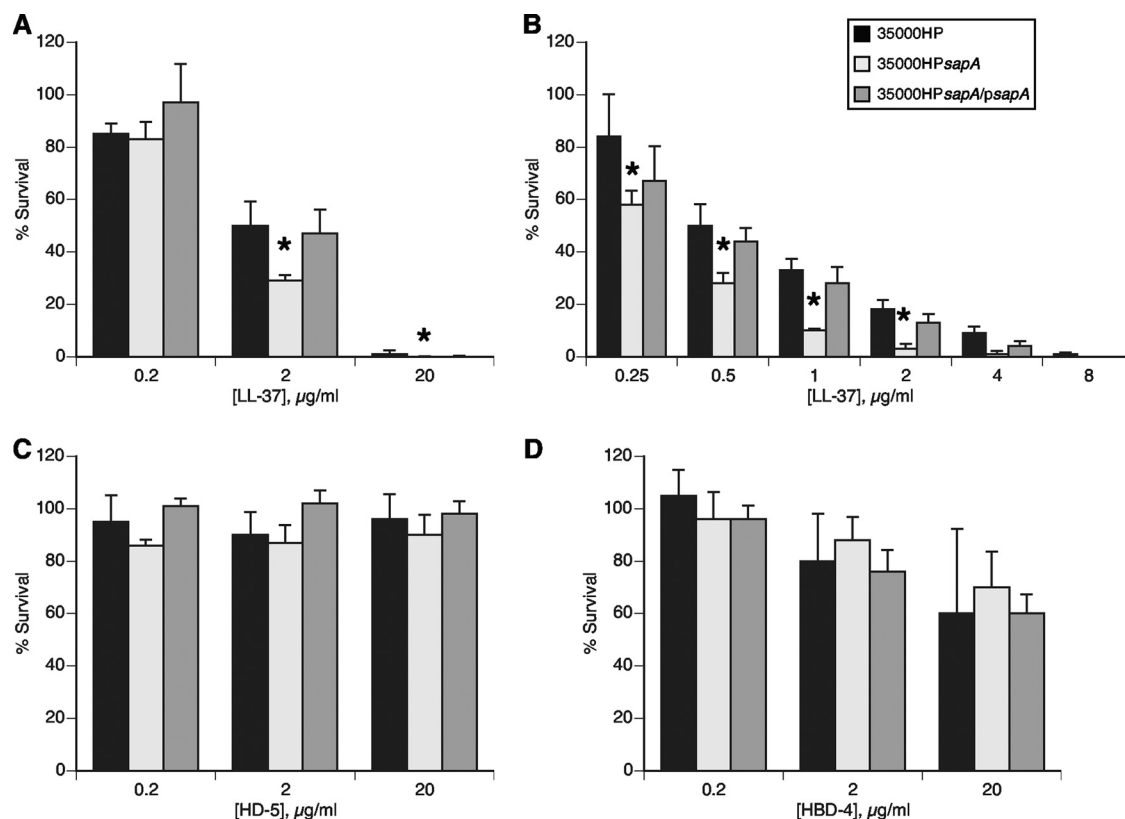


FIG. 3. *H. ducreyi* SapA confers resistance to the human cathelicidin LL-37, but not to human α - or β -defensins. Bactericidal assays comparing the percent survival of *H. ducreyi* 35000HP, 35000HPsapA, and 35000HPsapA/psapA exposed to the indicated concentrations of LL-37 (A and B), α -defensin HD-5 (C), or β -defensin HBD-4 (D). Data represent the means plus standard errors (error bars) for three independent assays. Percent survival values that are significantly different ($P \leq 0.01$) from those of the parent strain are indicated by an asterisk.

inoculated sites and at only 1 of 9 mutant-inoculated sites (Table 3).

The two iterations were combined for a statistical comparison of the strains (see Materials and Methods). Results showed no differences between the papule formation rates at parent-inoculated sites (100% [95% CI, 60.6 to 100%]) and at mutant-inoculated sites (94.4% [95% CI, 83.9 to 99.9%]) ($P = 0.14$); day 1 papule sizes were also similar between the parent-inoculated sites ($24.6 \pm 14.1 \text{ mm}^2$) and mutant-inoculated sites ($26.1 \pm 23.3 \text{ mm}^2$) ($P = 0.70$). However, pustules formed at 66.7% (95% CI, 44.9 to 88.4%) of 18 parent-inoculated sites and at 33.3% (95% CI, 11.6 to 55.1%) of 18 mutant-inoculated sites ($P = 0.007$). Thus, although papule formation was not affected, expression of *sapA* had a significant effect on the ability of *H. ducreyi* to progress to pustule formation in humans. Although we are precluded by biosafety regulations from testing complemented mutants in humans, the data suggest that SapA contributes significantly to the virulence of *H. ducreyi* in humans.

Surface cultures taken daily during infection were positive for *H. ducreyi* at 67% of parent-inoculated and 44% of mutant-inoculated sites. At the clinical endpoint, 3 parent-inoculated and 3 mutant-inoculated sites were biopsied and cultured; *H. ducreyi* was recovered from 2 parent biopsy specimens and 3 mutant biopsy specimens. All colonies recovered from surface cultures ($n = 370$ from parent sites; $n = 200$ from mutant sites)

and biopsy specimens ($n = 71$ from parent sites; $n = 55$ from mutant sites), as well as colonies from the parent ($n = 62$) and mutant ($n = 72$) inocula, were screened for susceptibility to Kan to confirm the presence of *H. ducreyi* 35000HP (Kan sensitive) or 35000HPsapA (Kan^r). All colonies had the expected kanamycin susceptibility phenotype, suggesting no cross-contamination had occurred between parent-inoculated and mutant-inoculated sites.

DISCUSSION

Pathogenic mechanisms involved in the ability of *H. ducreyi* to survive *in vivo* are not well defined. Our previous research demonstrated that class I and class II *H. ducreyi* strains resist killing by several human APs and suggested that this phenotype may be an important survival mechanism during infection (38). In this study, we characterized the *H. ducreyi* Sap operon and generated an isogenic *sapA* mutant in *H. ducreyi* 35000HP. Using this mutant, we have demonstrated a role *in vitro* for SapA in *H. ducreyi* resistance to the cathelicidin LL-37; we also defined SapA as an important virulence factor of *H. ducreyi* in human disease. To our knowledge, this is the first study to show that an AP resistance mechanism contributes to bacterial virulence in humans.

Sap transporters have been shown to be required for AP resistance in various Gram-negative pathogens of both an-

TABLE 3. Effect of *sapA* on human inoculation with *H. ducreyi*^a

| Volunteer (gender) | No. of days of observation | Strain ^b | Dose (CFU) ^c | No. of initial papules | No. of initial pustules | Final outcome of site | | |
|--------------------|----------------------------|---------------------|-------------------------|------------------------|-------------------------|-----------------------|-----------------|---------------------------------------|
| | | | | | | No. of papules | No. of pustules | No. of papules/pustules that resolved |
| 346 (male) | 6 | P | 81 | 3 | 3 | | 3 | |
| | | M | 42–165 | 3 | 2 | | 2 | 1 |
| 347 (male) | 6 | P | 81 | 3 | 2 | | 2 | 1 |
| | | M | 42–165 | 3 | 3 | | 3 | |
| 348 (male) | 7 | P | 81 | 3 | 1 | 1 | 1 | 1 |
| | | M | 42–165 | 3 | 0 | | | 3 |
| 349 (female) | 6 | P | 97 | 3 | 3 | | 3 | |
| | | M | 47–188 | 3 | 1 | | 1 | 2 |
| 350 (male) | 6 | P | 97 | 3 | 2 | | 2 | 1 |
| | | M | 47–188 | 3 | 0 | | | 3 |
| 351 (female) | 7 | P | 97 | 3 | 1 | | 1 | 2 |
| | | M | 47–188 | 2 | 0 | | | 2 |

^a Volunteers 346, 347, and 348 were inoculated in the first iteration; volunteers 349, 350, and 351 were inoculated in the second iteration. See the text for details.

^b P, parent strain 35000HP; M, mutant strain 35000HP*sapA*.

^c 42–165, one dose each of 42, 84, and 165 CFU; 47–188, one dose each of 47, 94, and 188 CFU.

imal and plant hosts. In *S. enterica* serovar Typhimurium, *sapABCD* mutants are more susceptible than the wild-type bacteria to the defensin melittin and to crude extracts of human granulocytes and contribute to virulence in mice (19, 41). In nontypeable *H. influenzae*, a *sapA* mutant confers resistance to the chinchilla beta defensin 1 (cBD-1) and to human APs HBD-3 and LL-37; the *sapA* mutant is also attenuated in a chinchilla model of infection (34, 35). A Sap transporter in the plant pathogen *Erwinia chrysanthemi* confers resistance to two classes of plant AP and contributes to virulence (32). McCoy et al. generated AP-susceptible mutants housing mutations within the *sap* operon of *Proteus mirabilis*; each of these mutants is susceptible to polymyxin B, and two are also susceptible to the synthetic protegrin IB-367 (36). Interestingly, while the Sap transporter plays a role in the AP resistance of many different bacteria, it does not confer AP resistance to all bacteria expressing *sap* genes. In *Vibrio fischeri*, a polar mutation within the *sapABCD* operon does not confer resistance to any of the eight APs tested, including LL-37, but it does affect *in vitro* growth and *in vivo* colonization of the host (33). Taken together, these studies indicate that the Sap transporter has evolved distinct functionality within each bacterial species to meet the need of that particular pathogen.

The contributions of Sap transporters to AP resistance of *H. ducreyi* and *H. influenzae* are not identical. *H. ducreyi* 35000HP*sapA* showed increased sensitivity to the human cathelicidin but not to α - or β -defensins; in contrast, the *H. influenzae sapA* mutant affects resistance to both cathelicidin and β -defensins (34, 35). Additionally, although the effect of the *H. ducreyi sapA* mutation on LL-37 resistance was significant, the roughly 25% reduction in survival (Fig. 3) was not as profound as the 8-fold reduction reported in *H. influenzae* (35). The lack of observed Sap-mediated effects on defensins in *H. ducreyi* suggests that the *H. ducreyi* and *H. influenzae* Sap transporters may differ in their specificities for APs. Whether this difference would be at the level of the AP binding SapA or at

the downstream step of transport through the Sap channel is unknown.

How Sap transporters function to protect the cell from AP attack is not clearly defined, although models have been proposed based on the homology of *sap* genes with genes of other ABC transporters (34, 41). Studies to compare the fate of APs in parent and isogenic *sap* mutant bacteria could shed light on the mechanism of Sap-mediated AP resistance. Because Sap is an uptake transporter, APs would be expected to be incorporated into the cell's cytoplasm or inner membrane, respectively, in parent and *sap* mutant bacteria, with differences being discerned by subcellular localization of the AP. In *H. ducreyi*, however, the modest difference in LL-37 susceptibility between strains 35000HP and 35000HP*sapA* suggests that we would observe only a minor difference in AP localization between the two strains, which is not likely to be informative. Thus, we did not pursue these experiments in our system.

Although the Sap transporter has a significant effect on LL-37 resistance in *H. ducreyi*, our findings also suggest that *H. ducreyi* expresses additional AP resistance mechanism(s) that function alongside the Sap transporter to thwart killing by defensins. Investigations with *S. Typhimurium* and *Staphylococcus aureus* have revealed the presence of several redundant resistance mechanisms within the same bacterial species (27). These redundant mechanisms seem to complement one another to achieve high-level resistance against a broad spectrum of APs. Sequence analysis of the *H. ducreyi* genome reveals the presence of regions of homology to other known AP resistance factors, suggesting that a similar dynamic may occur within *H. ducreyi*. We are currently examining the roles of these other putative resistance factors in *H. ducreyi*.

Our previous work examining *H. ducreyi* transcripts *in vivo* identified several *sap* genes and suggested that the *sap* operon may be upregulated during human infection. Neither the regulator nor an *in vivo* signal has been identified for upregulation of the *sap* operon. We examined the ability of LL-37 to act as

a regulatory signal but observed no transcriptional response of *sapA* to LL-37. We also examined the effects of *cpxRA* genes, the only intact two-component regulator in *H. ducreyi*, and saw no effect on *sapA* expression. Interestingly, the first gene in the *sap* operon is a homolog of *tyrR*, the gene product of which regulates aromatic amino acid synthesis and transport in *E. coli* (42). Whether the *sap* operon is regulated by TyrR is under investigation.

Sequence analysis demonstrated that the *sapA* ORF is present in both class I and class II *H. ducreyi* strains. As has been shown with other *H. ducreyi* virulence factors, we found a minor variation in the ORFs of class I strains but relatively greater variation between *sapA* ORFs of class I and class II strains (9, 51). The conservation of SapA among class I and class II clinical isolates of *H. ducreyi*, coupled with the conserved AP resistance phenotype (38), suggests that SapA-mediated AP resistance may be a conserved mechanism in *H. ducreyi*. PCR amplification using primers specific to other genes in the *sapABCD* operon further suggested that the operon structure may be conserved among class I and class II strains (data not shown).

The human model of *H. ducreyi* infection is a powerful tool for discerning genes that contribute to *H. ducreyi* pathogenesis (25). In this study, we used the human challenge model to show that SapA contributes significantly to human disease caused by *H. ducreyi*. Although these studies do not demonstrate the mechanism for attenuation of the *sapA* mutant *in vivo*, our *in vitro* data suggest that SapA may be important *in vivo* for resisting LL-37-mediated killing. An AP resistance mechanism contributing to *in vivo* survival is consistent with our understanding of *H. ducreyi* pathogenesis, in that the organism is surrounded by LL-37-secreting cells during infection (8, 10). The partial resistance to LL-37 *in vitro* correlates with the partial attenuation *in vivo*. Future studies will include determining whether loss of the Sap channel proteins leads to a greater phenotype *in vitro* and *in vivo*.

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