

# Loss of a Biofilm-Inhibiting Glycosyl Hydrolase during the Emergence of *Yersinia pestis*<sup>∇</sup>

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***Yersinia pestis*, the bacterial agent of plague, forms a biofilm in the foregut of its flea vector to produce a transmissible infection. The closely related *Yersinia pseudotuberculosis*, from which *Y. pestis* recently evolved, can colonize the flea midgut but does not form a biofilm in the foregut. *Y. pestis* biofilm in the flea and in vitro is dependent on an extracellular matrix synthesized by products of the *hms* genes; identical genes are present in *Y. pseudotuberculosis*. The *Yersinia Hms* proteins contain functional domains present in *Escherichia coli* and *Staphylococcus* proteins known to synthesize a poly- $\beta$ -1,6-*N*-acetyl-D-glucosamine biofilm matrix. In this study, we show that the extracellular matrices (ECM) of *Y. pestis* and staphylococcal biofilms are antigenically related, indicating a similar biochemical structure. We also characterized a glycosyl hydrolase (NghA) of *Y. pseudotuberculosis* that cleaved  $\beta$ -linked *N*-acetylglucosamine residues and reduced biofilm formation by staphylococci and *Y. pestis* in vitro. The *Y. pestis* *nghA* ortholog is a pseudogene, and overexpression of functional *nghA* reduced ECM surface accumulation and inhibited the ability of *Y. pestis* to produce biofilm in the flea foregut. Mutational loss of this glycosidase activity in *Y. pestis* may have contributed to the recent evolution of flea-borne transmission.**

The plague bacillus *Yersinia pestis* is maintained in flea-rodent transmission cycles in many parts of the world. Plague transmission is facilitated by the ability of *Y. pestis* to form a bacterial biofilm on the spines that line the interior surface of the flea's proventriculus, a valve guarding the entrance to the midgut that opens and closes rhythmically during blood feeding (23, 29). Growth and consolidation of the biofilm among the proventricular spines interfere with the patency of the proventricular valve and the passage of blood into the midgut, resulting in regurgitative transmission of bacteria into the bite site when an infected flea attempts to feed (2, 3). Eventually, the proventriculus can become completely blocked with *Y. pestis* biofilm. Blocked fleas make frequent, persistent attempts to take a blood meal before they die from starvation, and this altered feeding behavior increases the chances of transmission.

A biofilm is a dense aggregate of microorganisms embedded in an extracellular matrix (ECM) and usually attached to a surface (10). Biofilm formation in the flea and in vitro is mediated by the *Y. pestis* *hms* gene products, which are responsible for an extracellular material produced at growth temperatures of  $\leq 26^\circ\text{C}$  (13, 23, 29, 40). Homologous genes in other bacteria, including the *ica* genes of *Staphylococcus aureus* and *Staphylococcus epidermidis* and the *pga* genes of *Escherichia coli*, act to synthesize an ECM of poly- $\beta$ -1,6-linked *N*-acetyl-D-glucosamine ( $\beta$ -1,6-GlcNAc) that is essential for biofilm formation (11, 21, 50).

Flea-borne transmission, as well as the increased virulence of *Y. pestis*, coevolved within the past 20,000 years, the time frame in which *Y. pestis* diverged from *Yersinia pseudotuberculosis*, a relatively benign food- and waterborne intestinal pathogen (1, 35). The close phylogenetic relationship between these two species is reflected in their high degree of genomic identity. For example, *Y. pestis* contains only 32 chromosomal genes that do not have identical or highly similar orthologs in *Y. pseudotuberculosis*, and the nucleotide sequences of the *hms* genes are identical in the two species (8, 39). Despite this, *Y. pseudotuberculosis* produces less-cohesive biofilms than *Y. pestis* under some in vitro conditions and never colonizes the flea proventriculus even though most strains produce a chronic infection in the midgut (16).

*Y. pestis* contains many pseudogenes and genes disrupted by insertion sequence elements that are intact genes in *Y. pseudotuberculosis*. This suggests that *Y. pestis* is in the early stages of genome decay, eliminating genes no longer needed to survive outside of its insect or mammalian hosts (8, 39, 51). One of the *Y. pseudotuberculosis* genes (YPTB1123) that appears to be a pseudogene in *Y. pestis* is predicted to encode a family 20 glycosyl hydrolase enzyme and was annotated as chitinase (*chb*) (8, 22, 39). Notably, another family 20 glycosyl hydrolase, dispersin B of *Actinobacillus*, cleaves poly- $\beta$ -1,6-GlcNAc and disrupts biofilm formation in *Y. pestis* and other bacteria containing genes similar to *hms* (28, 31). Given the importance of biofilm production to *Y. pestis* transmission, we hypothesized that mutational loss of *chb* was selectively favored during the evolution of *Y. pestis* from *Y. pseudotuberculosis* because it resulted in a more stable biofilm in the flea vector. In this study, we demonstrated that the structures of the Hms-dependent ECM and the poly- $\beta$ -1,6-GlcNAc ECM of *S. epidermidis* are similar and characterized the YPTB1123 gene product as a

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TABLE 1. Comparison of putative glycosidases for the annotated genomes of *Y. pseudotuberculosis* IP32953 and *Y. pestis* CO92<sup>a</sup>

Family	Predicted enzyme(s)	<i>Y. pstb</i> ORF	<i>Y. pestis</i> ORF	Gene name
1	$\beta$ -Glucosidase, $\beta$ -galactosidase, $\beta$ -mannosidase	YPTB1290	YPO1254	<i>bglA</i>
2	$\beta$ -Galactosidase	YPTB2415	YPO1654	<i>lacZ</i>
3	$\beta$ - <i>N</i> -Acetylhexosaminidase, $\beta$ -glucosidase	YPTB1055	YPO2803	<i>bglB</i>
	$\beta$ - <i>N</i> -Acetylhexosaminidase, $\beta$ -glucosidase	YPTB2449	YPO1615	<i>ycfO/nagZ</i>
	$\beta$ - <i>N</i> -Acetylhexosaminidase, $\beta$ -glucosidase	YPTB3439	YPO0616	<i>bglX</i>
4	$\alpha$ -Glucosidase, $\alpha$ -galactosidase, 6P- $\beta$ -glucosidase	YPTB3735	YPO0166	<i>chbF</i>
5	<b>Chitosanase, cellulase, endo-1,6-<math>\beta</math>-glucosidase, endo-1,6-<math>\beta</math>-galactanase</b>	<b>YPTB3269</b>	<b>YPO0757 IS insertion</b>	
8	<b>Chitosanase, cellulase, endo-1,4-<math>\beta</math>-xylanase</b>	<b>YPTB3837</b>	<b>YPO3998 frameshift</b>	<i>bcsZ</i>
13	$\alpha$ -Amylase	YPTB3786	YPO3941	<i>glgX</i>
	1,4- $\alpha$ -Glucan branching	YPTB3787	YPO3942	<i>glgB</i>
	Trehalose-6-phosphate hydrolase	YPTB3537	YPO3696	<i>treC</i>
	$\alpha$ -Amylase	YPTB3909	YPO4080	<i>malS</i>
	Maltodextrin glucosidase	YPTB0922	YPO3200	<i>malZ</i>
18	<b>Chitinase, endo-<math>\beta</math>-<i>N</i>-acetylglucosaminidase</b>	<b>YPTB3365</b>	<b>Absent</b>	<i>chiC</i>
20	<b><math>\beta</math>-Hexosamidase</b>	<b>YPTB1123</b>	<b>YPO2632 frameshift</b>	<i>chb (nghA)</i>
23	Lysozyme, type G	YPTB0595	YPO0452	<i>slt</i>
	Lysozyme, type G	YPTB2346	YPO2438	<i>mltC</i>
	Lysozyme, type G	YPTB2681	YPO2957	<i>mltB</i>
	Lysozyme, type G	YPTB2880	YPO2922	<i>slt</i>
	Lysozyme, type G	YPTB2968	YPO1078	<i>mltD</i>
	Lysozyme, type G	YPTB3226	YPO0954	<i>mltC</i>
24	Lysozyme	YPTB1755	YPO2098	
	<b>Lysozyme</b>	<b>YPTB1805</b>	<b>Absent</b>	
31	$\alpha$ -Glucosidase	YPTB3093	YPO0848	
36	$\alpha$ -Galactosidase	YPTB1608	YPO1581	<i>rafA</i>
42	$\beta$ -Galactosidase	YPTB3097	YPO0852	<i>bgA</i>
53	Endo-1,4- $\beta$ -galactanase	YPTB3098	YPO0853	
73	Endo- $\beta$ - <i>N</i> -acetylglucosaminidase, <i>N</i> -acetylmuramoylhydrolase	YPTB1680	YPO1807	<i>flgJ</i>
77	4- $\alpha$ -Glucanotransferase	YPTB3774	YPO0126	<i>malQ</i>
94	Cellobiose phosphorylase	YPTB3445	YPO0610	

<sup>a</sup> Genome data were compiled using the carbohydrate-active-enzyme server (<http://www.cazy.org>). Genes predicted to be absent or nonfunctional in *Y. pestis* are highlighted in bold. *Y. pstb*, *Y. pseudotuberculosis*; ORF, open reading frame.

glycosyl hydrolase whose activity interferes with biofilm formation.

#### MATERIALS AND METHODS

**Bacterial strains.** *Y. pestis* KIM6+ and KIM6 (19), *Y. pseudotuberculosis* IP32953 (8), and *S. epidermidis* 1457 and its isogenic Ica<sup>-</sup> derivative (36) were used. *Y. pestis* KIM6 is an Hms<sup>-</sup> derivative of KIM6+ that lacks the Pgm locus that contains the *hmsHSFR* operon. Both strains lack the pYV virulence plasmid, which is not required to produce a transmissible infection in fleas (23).

**Immunofluorescence assays.** *Y. pestis* KIM6+ and KIM6 were grown on heart infusion agar (Difco) containing 0.2% galactose at 21 and 37°C. Wild-type and Ica<sup>-</sup> *S. epidermidis* bacteria were grown in tryptic soy broth at 37°C. Bacterial suspensions were prepared after 24 h, spotted on glass slides, allowed to air dry, and incubated with a 1:100 dilution of polyclonal antiserum generated against purified Ica-dependent ECM polysaccharide (PIA) of *S. epidermidis*, from which nonspecific antibodies had been removed by absorption with whole bacteria and cell extracts of Ica<sup>-</sup> *S. epidermidis* (49). After 1 h of incubation at 37°C, the slides were washed and reincubated with a 1:50 dilution of fluorescein isothiocyanate-labeled goat antirabbit secondary antibody (Pierce Biotechnology). One hour later, the slides were washed and redried before examination by fluorescence and phase-contrast microscopy.

**Cloning and deletion of YPTB1123 (*ngH4*).** The predicted *ngH4* open reading frame including upstream and downstream regions was PCR amplified from *Y. pseudotuberculosis* IP32953 genomic DNA using the primers 5'-GCA CTA CAC CCT GTA TGA CAA CC-3' and 5'-GCG TTA CAC ATC AGG CTG TTG-3' with *Pfu* polymerase (Stratagene). The PCR product was cloned into the high-copy-number pCR4Blunt cloning vector (Invitrogen; ~200 copies per cell) to generate the plasmid pCR4Blunt<sup>ngH4</sup> and into the low-copy-number (6 to 8 copies per cell) pLG338 cloning vector (44) to generate the plasmid pLG338<sup>ngH4</sup>. Each plasmid was used to transform *Y. pestis* KIM6+ via electroporation. The entire open reading frames plus upstream and downstream regions of other *Y. pseudotuberculosis*-specific glycosyl hydrolases (Table 1) were cloned into the pCR4Blunt plasmid.

A 2.2-kb deletion from pCR4Blunt<sup>ngH4</sup> that eliminated a 2.2-kb internal fragment of the *ngH4* gene was generated by inverse PCR using the primers 5'-CCG CTT GCC AGT ACC GGG GGC CAG AGT GGT GGC-3' and 5'-GCC CAG TCC TGG CCT TTG ATC TCA TCC CCG GTG-3' and religation. A fragment containing the deleted allele was subcloned into the suicide vector pCVD442 (15), which was then introduced into *E. coli* S17-1. The suicide vector construct was transferred to *Y. pseudotuberculosis* IP32953 by conjugation. *Y. pseudotuberculosis* transconjugants in which allelic exchange had occurred were PCR tested to verify deletion of *ngH4*.

**Colloidal gold labeling.** Silicon chips were applied briefly to bacterial lawns after 24 h of growth at room temperature. Adherent bacteria on the chips were washed with phosphate-buffered saline (PBS) and blocked with 1% bovine serum albumin-PBS for 2 min in a Pelco 3451 laboratory microwave oven (Ted Pella Inc., Redding CA) at 150 W, 37°C under vacuum (all additional steps were carried out with the same microwave settings). Samples were incubated in the microwave with anti-PIA antiserum diluted 1:100 in blocking buffer two times (2 min each), washed with blocking solution three times (1 min each), and incubated with goat antirabbit 10-nm colloidal gold (BBInternational, Cardiff, United Kingdom) diluted 1:100 two times (2 min each) before rinsing three times (1 min each) with PBS and once for 1 min with distilled H<sub>2</sub>O.

**Scanning electron microscopy.** Samples were prepared as described previously (18) with the following modifications. Immunogold-labeled samples were fixed twice (2 min each) at 150 W at 24°C under vacuum with 75 mM lysine acetate, 0.075% (wt/vol) alcian blue, 2% paraformaldehyde, and 2.5% glutaraldehyde, buffered with 0.1 M sodium cacodylate. Samples were washed two times (3 min each) with 0.1 M sodium cacodylate, and postfixed with 3 rounds of 1% osmium tetroxide–0.8% potassium ferricyanide in 0.1 M sodium cacodylate, alternating with two rounds of 1% tannic acid in distilled H<sub>2</sub>O for bulk conductivity (two times, 2 min for each round) at 80 W and 24°C under vacuum in the microwave oven. Samples were washed once for 5 min with 0.1 M sodium cacodylate and twice for 5 min with distilled H<sub>2</sub>O prior to dehydration with a graded ethanol series of 50%, 75%, and 95% and three with 100% for 40 s each at 80 W and 24°C. Samples were critical-point-dried under

CO<sub>2</sub> with a Bal-Tec cpd 030 drier (Balzers, Liechtenstein) and mounted on aluminum studs. Samples for backscatter imaging were left uncoated, and samples for secondary imaging were sputter coated with 35 Å of iridium in an IBS/TM200S ion beam sputterer (VCR Group, South San Francisco, CA) prior to viewing at 5 kV on a Hitachi S-5200 field emission in-lens scanning electron microscope in either backscatter composite or secondary imaging modes (Hitachi, Tokyo, Japan).

**Expression and purification of NghA.** To express NghA, the open reading frame was PCR amplified using the primers 5'-ATG AGG TTA GTA ATG AAC AAA T-3' and 5'-GAT GTT AAC TGG CTC GGC ACG-3' and the product cloned into the pBAD-Thio-HIS cloning vector (Invitrogen), which adds a C-terminal six-His tag. *E. coli* TOP10 cells (Invitrogen) containing the plasmid were grown in 500 ml of Luria broth, induced with 0.2% arabinose at mid-log phase, and grown overnight at 28°C. Recombinant His-tagged protein was purified from the cell pellet under native conditions using nickel-nitrilotriacetic acid columns (Qiagen) according to the instructions of the manufacturer. The purity of the eluted protein was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie blue staining. Protein concentrations were determined by using the BCA protein assay kit (Pierce).

**Glycosyl hydrolase assays.** The synthetic substrates 4-nitrophenyl-*N*-acetyl-β-D-glucosaminide, 4-nitrophenyl-*N*-acetyl-β-D-galactosaminide, 4-nitrophenyl-*N*-acetyl-α-D-glucosaminide, and 4-nitrophenyl-*N*-acetyl-α-D-galactosaminide (Sigma) were dissolved in 50 mM sodium phosphate buffer (pH 5.9) at a 5 mM final concentration (31). Nine hundred microliters of substrate solution was added to 100 μl buffer containing 4 μg purified NghA protein. After incubation at 37°C for 0 to 80 min, reactions were stopped by adding 5 μl 10 N NaOH. To determine inhibitory effects of excess glucose, *N*-acetylglucosamine, chitobiose, or staphylococcal PIA, 100 μl of 4-nitrophenyl-*N*-acetyl-β-D-glucosaminide substrate was added to 100 μl enzyme solution containing 4 μg purified NghA with or without 0.4% glucose or *N*-acetylglucosamine; 10 mM *N,N'*-diacetylchitobiose (Sigma); or 10 mg PIA. PIA was prepared from *S. epidermidis* 1457 as described previously (48) by heating cell pellets at 100°C for 10 min in 0.5 M EDTA, pH 8.0, centrifugation, dialysis of the supernatant, and lyophilization. *Y. pestis* and *Y. pseudotuberculosis* cell extracts were prepared by centrifuging 1 ml bacterial culture containing  $\sim 1.0 \times 10^8$  bacteria/ml, resuspending the cell pellets in 500 μl sodium phosphate reaction buffer, adding 20 μl of 0.2% sodium dodecyl sulfate and 40 μl chloroform, and vortexing for 10 s. For enzyme assays, 100 μl of the cell extract mixture was added to 100 μl of 4-nitrophenyl-*N*-acetyl-β-D-glucosaminide substrate solution. Reactions with excess glucose, *N*-acetylglucosamine, chitobiose, or PIA and with cell extracts were stopped by adding 800 μl 1 M NaCO<sub>3</sub>. Enzymatic activity (release of the 4-nitrophenyl indicator group) was measured spectrophotometrically at 405 nm. The absorbance at 550 nm was also measured, as was the absorbance at 600 nm of the original culture from which the extract was derived. These values were used to calculate β-hexosaminidase units in the cell extracts, where 1 unit equals  $10,000 \times \{[A_{405} - (1.75 \times A_{550})]/(\text{time} \times A_{600})\}$  (reaction time in minutes).

**In vitro biofilm assays.** To determine the ability of NghA to inhibit biofilm formation, purified recombinant NghA was concentrated in TMH liquid medium (46) without gluconate using a Millipore 30,000-molecular-weight-cutoff filter. Biofilm formation or dispersal of *S. epidermidis* and *Y. pestis* KIM6+ in 96-well polystyrene microtiter plates was measured (28) in the presence or absence of 50 μg/ml NghA. *Y. pestis* biofilms were grown in TMH with 0.2% galactose at 21°C. The adherent biofilm was washed three times with water, stained with safranin, and measured spectrophotometrically at 490 nm (16). *S. epidermidis* biofilms were grown in tryptic soy broth at 37°C, stained with crystal violet, washed three times with water, and measured at 605 nm. Biofilms were measured in three wells at each time point. To determine the effect of NghA expression, biofilms of *Y. pestis* KIM6+ carrying the plasmid pLG338, pCR4Blunt, pLGnghA, or pCRBnghA were grown in microtiter plates in TMH medium containing 10 μg/ml hemin and measured as described above. Quantitative differences between strains were assessed by using a paired, two-tailed *t* test.

**Flea infections.** Fleas were infected by allowing them to feed on heparinized mouse blood containing  $1 \times 10^8$  to  $5 \times 10^8$  *Y. pestis*/ml in a membrane feeder device (16, 23). Fleas (50 females and 50 males) were maintained for 28 days and checked twice weekly for proventricular blockage (presence of fresh blood in the esophagus but not in the midgut) immediately after they had been allowed to feed on an uninfected mouse. Additional samples of 20 female fleas from each experiment were collected at 0, 7, and 28 days after the infectious blood meal to determine the percentage of infected fleas (16, 23). Three or four independent infection experiments were done with each strain.

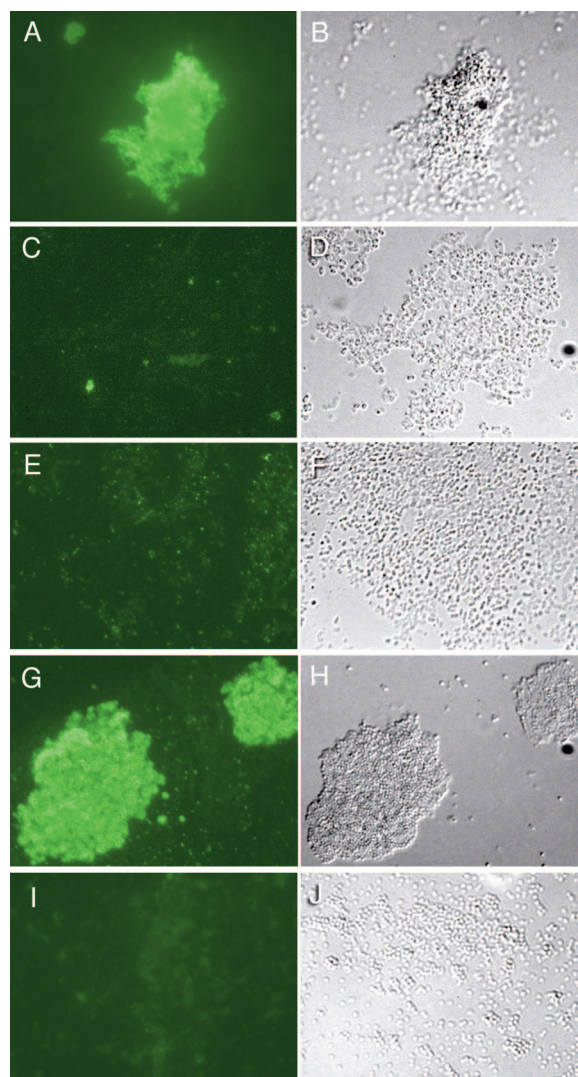


FIG. 1. Antibody to the poly-β-1,6-*N*-acetylglucosamine ECM of *S. epidermidis* biofilm cross-reacts with *Y. pestis* ECM. Immunofluorescence assays of Hms<sup>+</sup> (A to D) or Hms<sup>-</sup> (E and F) *Y. pestis* or Ica<sup>+</sup> (G and H) or Ica<sup>-</sup> (I and J) *S. epidermidis* using polyclonal antibody generated against purified Ica-dependent ECM polysaccharide of *S. epidermidis* are shown. *Y. pestis* strains were cultured at 21°C (A, B, E, and F) or 37°C (C and D); the Hms-dependent ECM is expressed only at 26°C or below (40). The matching phase-contrast image is shown to the right of each immunofluorescence image. Magnification, ×600.

## RESULTS

**Antigenic cross-reactivity of the ECM of *Yersinia* and staphylococcal biofilms.** The chemical structure of the *Y. pestis* Hms-dependent ECM has not been defined, but the similarity between the *hms*, *ica*, and *pga* genes suggests that it too is a β-1,6-GlcNAc polymer. To assess the biochemical relatedness of the *Yersinia* and *S. epidermidis* ECMs, we tested whether antibody raised against PIA, the Ica-dependent β-1,6-GlcNAc ECM of *S. epidermidis* biofilm, would cross-react with the Hms-dependent ECM of *Y. pestis*. The anti-PIA antiserum reacted strongly with the *Y. pestis* strain containing the *hms* genes grown at 21°C but not at 37°C (Fig. 1A to D). The antibody did not bind to the Hms<sup>-</sup> *Y. pestis* strain, which is

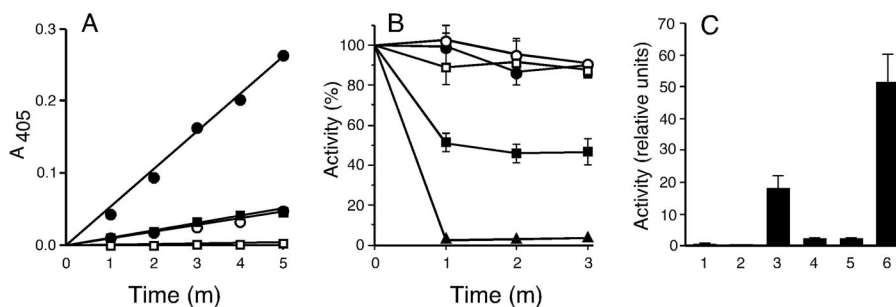


FIG. 2. *Y. pseudotuberculosis* NghA is a family 20 glycosyl hydrolase with highest activity against  $\beta$ -*N*-acetylglucosamine linkages and an affinity for chitobiose. (A) Release of 4-nitrophenyl (NP) adduct from 4-NP-*N*-acetyl- $\beta$ -D-glucosaminide (filled circles), 4-NP-*N*-acetyl- $\alpha$ -D-glucosaminide (open circles), 4-NP-*N*-acetyl- $\beta$ -D-galactosaminide (filled squares), or 4-NP-*N*-acetyl- $\alpha$ -D-galactosaminide (open squares) was measured during incubation at 37°C with purified recombinant NghA at the indicated times. (B) Release of 4-NP by NghA from 4-NP-*N*-acetyl- $\beta$ -D-glucosaminide in the presence of 0.4% glucose (open squares), 10 mM *N*-acetylglucosamine (closed squares), 10 mM chitobiose (triangles), or 20 mg/ml PIA extract from wild-type *S. epidermidis* (circles). (C) Hydrolysis of 4-NP-*N*-acetyl- $\beta$ -D-glucosaminide by cell extracts of *Y. pestis* grown at 21°C (1) or 37°C (2), *Y. pseudotuberculosis* grown at 21°C (3) or 37°C (4), or *Y. pestis* containing the plasmids pLGnghA (5) or pCRBnghA (6) grown at 21°C. Relative  $\beta$ -hexosaminidase units were calculated as described in Materials and Methods. Results in panels A and C are the means  $\pm$  standard deviations of a representative experiment performed in triplicate; results in panel B are the means  $\pm$  standard errors of the means for three independent experiments performed in triplicate.

unable to form biofilm or colonize the proventriculus of fleas, at either temperature (Fig. 1E and F). This result is highly suggestive that the biofilm ECM of the two species have similar poly- $\beta$ -1,6-GlcNAc-based structures.

**The *Y. pseudotuberculosis* YPTB1123 gene encodes a  $\beta$ -*N*-acetylglucosaminidase.** *Y. pseudotuberculosis*, like *Y. pestis*, forms robust Hms-dependent biofilms in many in vitro environments and on the outer mouthparts of *Caenorhabditis elegans* (13, 30). However, *Y. pseudotuberculosis* never forms biofilm on the flea proventriculus to cause blockage of the digestive tract, even though it can colonize the flea midgut (16). We reasoned that the inability of *Y. pseudotuberculosis* to colonize the proventriculus could in part be due to alteration or instability of the  $\beta$ -1,6-GlcNAc ECM polymer. The predicted amino acid sequence of the *Y. pseudotuberculosis* YPTB1123 gene product contains the signature carbohydrate-binding and catalytic domains of family 20 glycosyl hydrolases, including the conserved glutamate and aspartate residues that are essential for activity (41). The protein also contains an N-terminal signal peptide sequence but no predicted transmembrane domains, indicating that it is most likely localized to the periplasm (6).

To determine whether YPTB1123 encodes a glycosyl hydrolase, we cloned this gene from *Y. pseudotuberculosis*, isolated recombinant protein, and tested it for its ability to hydrolyze various glycosidic linkages of 4-nitrophenyl-labeled synthetic hexosamine substrates. The protein had greatest hydrolytic activity against *N*-acetyl- $\beta$ -D-glucosaminide, less activity against *N*-acetyl- $\beta$ -D-galactosaminide and *N*-acetyl- $\alpha$ -D-glucosaminide, and no activity against *N*-acetyl- $\alpha$ -D-galactosaminide (Fig. 2A).  $\beta$ -D-Glucosaminidase activity was strongly inhibited by the addition of chitobiose to the reaction and to a lesser extent by GlcNAc but not by glucose (Fig. 2B). Extracts enriched for staphylococcal PIA did not inhibit enzymatic activity (Fig. 2B), suggesting that the active site of the enzyme has less affinity for the mature  $\beta$ -1,6-*N*-acetyl-D-glucosamine PIA polymer, which is substantially deacetylated (49). These results are consistent with the activity of a  $\beta$ -*N*-acetylhexosaminidase with affinity for chitobiose; we there-

fore refer to YPTB1123 as *nghA* ( $\beta$ -*N*-acetyl-D-glucosamine hydrolase A).

***Y. pseudotuberculosis* NghA inhibits *Yersinia* and staphylococcal biofilm formation.** The enzymatic activity of NghA suggested it might interfere with production or stability of the ECM. We first tested whether *Y. pseudotuberculosis* produces detectable  $\beta$ -hexosaminidase activity. Lysates of *Y. pseudotuberculosis* grown at 21°C had higher  $\beta$ -hexosaminidase activities than those from cultures grown at 37°C (Fig. 2C). Our goal was to determine whether restoration of a functional *nghA* gene to *Y. pestis* results in altered ECM production and decreased biofilm formation in vitro or in vivo. For these experiments, *Y. pestis* was transformed with pCRBnghA or pLGnghA, recombinant high- and low-copy-number plasmids, respectively, that contain the *Y. pseudotuberculosis* *nghA* gene and promoter region. The high-copy-number plasmid conferred approximately 2.5-fold-greater  $\beta$ -hexosaminidase activity to *Y. pestis* than *Y. pseudotuberculosis*, whereas the low-copy-number plasmid resulted in a level approximately fourfold lower than that of *Y. pseudotuberculosis* and the wild-type (*NghA*<sup>-</sup>) *Y. pestis* parent strain had none (Fig. 2C).

We then tested the effect of *nghA* restoration on *Y. pestis* ECM by immunoelectron microscopy using gold-labeled anti-PIA antibody. The majority of wild-type cells were extensively labeled with gold particles (Fig. 3A). Secondary imaging gave a higher resolution of the surface morphology independently of the gold particles; these images showed that the antibody-reactive Hms-dependent material was associated primarily with extensive bumps on the surface of the bacteria (Fig. 3B). In contrast, Hms<sup>-</sup> cells were uniformly unlabeled and smooth (Fig. 3C and D). Restoration of *nghA* resulted in reduced, more variable immunogold labeling, with some indication of possible sloughing of the Hms-dependent matrix (Fig. 3E and G). This was accompanied by reduced cell surface ECM (Fig. 3F and H), a consistent finding that correlated with the *nghA* copy number—the surface of the bacteria carrying the high-copy vector appeared smooth, much like the Hms<sup>-</sup> strain (Fig. 3D and H).

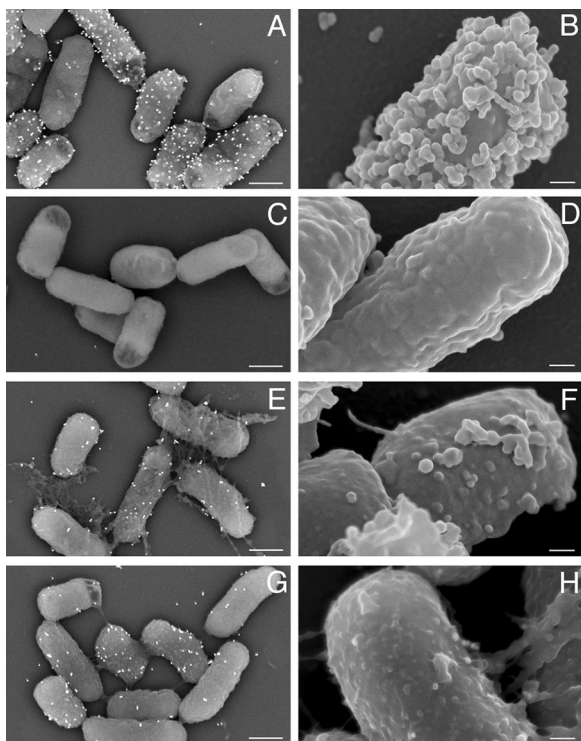


FIG. 3. NghA expression prevents accumulation of Hms-dependent ECM. *Y. pestis* KIM6+ (Hms<sup>+</sup>) (A and B), KIM6 (Hms<sup>-</sup>) (C and D), or *Y. pestis* KIM6+ containing plasmid pLGnghA (E and F) or pCRBnghA (G and H) were assayed with gold-labeled anti-PIA antibody and analyzed by scanning electron microscopy in backscatter composite imaging mode (left panels) or secondary imaging mode (right panels). Scale bar = 0.5 μm (left panels) or 0.1 μm (right panels).

In vitro biofilm production of the different strains was evaluated in polystyrene microtiter plates. At 24 h, there were no differences in the biofilms formed by NghA<sup>+</sup> strains or the wild-type (NghA<sup>-</sup>) strains containing empty vectors. However, biofilms of *Y. pestis* overexpressing NghA (high copy number) measured at 48 and 72 h were significantly reduced ( $P < 0.0001$ ) compared to those of *Y. pestis* expressing less NghA (low copy number) or no NghA (empty vectors) (Fig. 4A).

To determine whether extracellular NghA impedes biofilm formation, we added it at  $t = 0$  to the *Y. pestis* and *S. epidermidis*

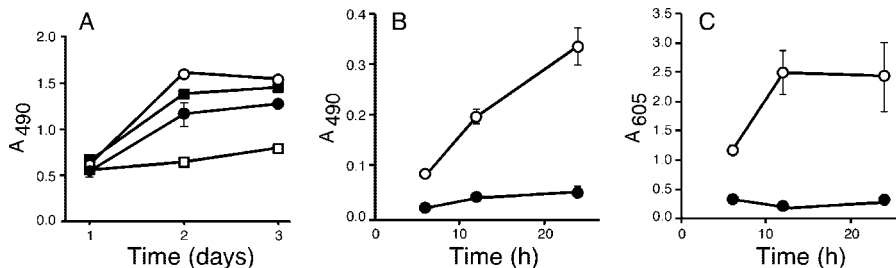


FIG. 4. NghA interferes with *Y. pestis* and *S. epidermidis* biofilm formation. (A) Quantitation of adherent biofilm produced by *Y. pestis* KIM6+ containing plasmid pCR4Blunt (filled squares), pLG338 (filled circles), pCRBnghA (open squares), or pLGnghA (open circles). (B and C) Quantitation of adherent biofilm produced by *Y. pestis* (B) or *S. epidermidis* (C) in triplicate wells of polystyrene microtiter plates in the presence (filled circles) or absence (open circles) of 50 μg/ml purified recombinant NghA. Results are the means ± standard deviations from two independent experiments (A) or a single representative experiment (B and C) performed in triplicate.

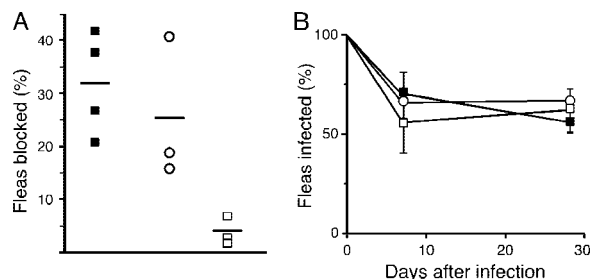


FIG. 5. Transformation of *Y. pestis* with functional *nghA* inhibits proventricular biofilm formation but not midgut colonization in fleas. (A) Percentage of fleas that developed proventricular blockage during a 28-day period after infection with *Y. pestis* (filled squares) or *Y. pestis* transformed with a high-copy-number (open squares) or low-copy-number (circles) plasmid containing *nghA*. Horizontal lines indicate means. (B) Flea midgut colonization rates (means ± standard errors of means) from experiments shown in panel A.

*midis* inocula in the microtiter plate quantitative biofilm assay. Similar to *Actinobacillus dispersin B* (28), exogenous NghA prevented in vitro biofilm formation for both species (Fig. 4B and C). However, adding NghA to preformed biofilms (after 24 or 48 h for *S. epidermidis* or *Y. pestis*, respectively) did not result in their disruption or dispersal (data not shown), consistent with the observation that the mature, presumably highly deacetylated form of the poly-β-1,6-*N*-acetyl-D-glucosamine ECM is not a competitive substrate for NghA (Fig. 2B).

**Overexpression of *Y. pseudotuberculosis* NghA in *Y. pestis* results in decreased proventricular blockage of fleas.** Biofilm development and maturation constitute a complex physiological process that is sensitive to environmental conditions and substrate characteristics (5). Therefore, we next measured the effect of NghA activity in the relevant biological context, the ability of *Y. pestis* to infect and produce a proventricular biofilm in the rat flea *Xenopsylla cheopis*, an important vector of plague worldwide (Fig. 5). The *Y. pestis* strain overexpressing the *nghA* gene (high copy number) blocked significantly fewer fleas than the wild-type strain (Fig. 5A). The mean blockage rate of fleas infected with the *Y. pestis* strain transformed with *nghA* on the low-copy-number plasmid was also reduced, but the difference was not statistically significant (Fig. 5A). Expression of functional *nghA* in *Y. pestis* did not affect the incidence of chronic digestive tract infection and the average bacterial load in fleas (Fig. 5B).

Although expression of functional *nghA* in *Y. pestis* appeared to destabilize biofilm formation in the flea, deletion of *nghA* was not sufficient by itself to enable *Y. pseudotuberculosis* to produce a stable biofilm in fleas. Fleas infected with a *Y. pseudotuberculosis*  $\Delta$ *nghA* strain never developed proventricular blockage, and this strain showed no defect in biofilm formation in polystyrene microtiter plates (data not shown). The *Y. pestis* and *Y. pseudotuberculosis* genomes contain additional genes that are predicted to encode glycosyl hydrolases of other families (Table 1). Four of these appear to be functional in *Y. pseudotuberculosis* but are pseudogenes or absent in *Y. pestis*. One encodes a phage-related lysozyme that is not likely to be active against the Hms-dependent ECM. The other three encode predicted family 5 and family 8 chitosanases or cellulases (YPTB3269 and YPTB3837) and a family 18 chitinase (YPTB3365). However, transformation of *Y. pestis* with individual plasmids containing these three genes did not affect biofilm formation in vitro or the ability to block fleas (data not shown).

## DISCUSSION

Our results provide further evidence that *Y. pestis* produces a transmissible infection in its flea vector by the same mechanism by which staphylococci infect catheters and other indwelling medical devices (21). In both cases, a poly- $\beta$ -1,6-GlcNAc ECM may enhance aggregation on a hydrophobic surface. The proventricular spines are covered with cuticle, the same acellular, hydrophobic material that makes up the insect exoskeleton. As predicted by the similarity between the ECM biosynthetic genes of staphylococci and yersiniae, anti-PIA antibody cross-reacted strongly with *Y. pestis* ECM (Fig. 1 and 3). Bobrov et al. (7) have reported similar evidence that the *S. epidermidis* and *Y. pestis* ECMs are structurally related  $\beta$ -1,6-GlcNAc polysaccharides. Both *Y. pestis* and *Y. pseudotuberculosis* are capable of forming in vitro biofilms, mediated by the ECM synthesized by their identical *hms* genes (13, 16, 30). However, only *Y. pestis* produces biofilm in the digestive tract of fleas. During the transition to its vector-borne lifestyle, therefore, *Y. pestis* adapted to grow as an adherent biofilm on proventricular cuticle. This adaptation could have involved alterations in environmental sensing, signal transduction, and biofilm gene regulation pathways; cell surface characteristics; or specific adhesins; all of which can have environment-specific effects on biofilm formation (5, 25, 45). In this study, we characterized an *N*-acetyl- $\beta$ -glucosaminidase enzyme from *Y. pseudotuberculosis*, showed that it interferes with ECM production, and examined whether its loss in *Y. pestis* contributed to the evolution of flea-borne transmission.

The *Y. pseudotuberculosis* YPTB1123 open reading frame was annotated as a putative chitobiase (*chb*) gene because its predicted amino acid sequence is highly similar to that of Chb of *Serratia marcescens* (39). In *Serratia* and other bacteria, Chb participates in the catabolism of chitin, in which extracellular chitinases release chitobiose and higher  $\beta$ -1,4-linked chitooligosaccharides that are then hydrolyzed by Chb to produce GlcNAc (32, 37). *E. coli* and other species are able to use chitobiose as a sole carbon source; the genes involved in chitobiose phosphotransferase uptake and utilization are encoded in the *chbBCARFG* operon (33). Both *Y. pseudotuberculosis* and *Y. pestis* contain an orthologous *chbBCARG* chitobiose

phosphotransferase uptake locus (*Y. pestis* YPO2678 to YPO2682), but the putative phosphochitobiase gene *chbF* (YPO0166) is not linked to this operon and is less similar to *E. coli chbF*. Interestingly, *Y. pseudotuberculosis* also contains an ortholog of one of the *S. marcescens* chitinases, *chiC*, that is absent in *Y. pestis* (Table 1). This suggests that the primary function of these genes in *Y. pseudotuberculosis* relates to degradation of environmental chitin and that their loss in *Y. pestis* was neutral because they were no longer required for the flea-mammal life cycle. However, our data indicate that YPTB1123 can prevent accumulation of the poly- $\beta$ -1,6-GlcNAc ECM of *Yersinia* biofilms, our rationale for referring to it by the more general Ngh descriptive. Thus, loss of *nghA* may have had the positive consequence of enhancing biofilm formation or stability in the flea and represent a selected rather than a neutral mutation. The orthologous gene in all five of the *Y. pestis* strains whose genome sequences have been reported contains a frameshift mutation after codon 182 and is predicted to encode a truncated, inactive protein (9, 14, 39, 43). Since the five strains are representative of the three major branches of the *Y. pestis* evolutionary tree (1), the gene loss appears to have occurred early in the divergence of *Y. pestis* from *Y. pseudotuberculosis*.

Although few studies have specifically examined synthesis and assembly of the *Yersinia* Hms ECM (7), synthesis of poly- $\beta$ -1,6 *N*-acetyl-D-glucosamine by staphylococci and *E. coli* and the functions of the Ica and Pga proteins are better understood. IcaA/PgaC (similar to HmsR) assemble short polymers from UDP-*N*-acetylglucosamine in the cytoplasm. In staphylococci, IcaD is also required for polymerization, and IcaC could link these polymers into longer chains and/or increase the processivity of the IcaAD enzyme (20). In *E. coli*, PgaD (similar to HmsS) is also localized to the inner membrane and likely assists PgaC in polymerization (27). The polymer is deacetylated by IcaB/PgaB (similar to HmsF), either at the cell surface after export in the case of staphylococci (47) or prior to export and transport through the outer membrane porin PgaA (similar to HmsH) in the case of *E. coli* (27). In *Y. pestis*, NghA could hydrolyze growing polymers at any point during their initial assembly in the cytoplasm, transport through the periplasm, or extracellular anchoring to the cell envelope, thereby reducing ECM levels and biofilm formation (Fig. 2 and 3). Purified NghA apparently has low affinity for fully formed (presumably deacetylated) PIA (Fig. 2) and does not destabilize already formed, mature *Y. pestis* or *S. epidermidis* biofilms yet is still able to prevent biofilm formation during growth (Fig. 4). One possible explanation for these results is that deacetylation or some other late modification of the polymer reduces its substrate affinity for NghA. Taken together, our results favor a model in which intracellular or secreted NghA interferes with subunit polymerization and accumulation, rather than degradation, of the ECM.

An effect of *nghA* function on *Y. pestis* biofilm was detected in the flea, but a significant reduction in flea blockage resulted only when *nghA* was present in high copy number (Fig. 5). This result is somewhat unexpected, since in *Y. pseudotuberculosis*, this gene is chromosomal and therefore occurs in a single copy. However, the levels of NghA activity conferred by a low-copy-number plasmid in *Y. pestis* were lower than those of wild-type *Y. pseudotuberculosis* under the conditions we tested (Fig. 2C),

suggesting that there may be pre- or posttranscriptional regulatory differences that reduce the amount of NghA made by *Y. pestis*. Additionally, previous experiments performed with *C. elegans* indicate a difference in the matrices made by *Y. pestis* and *Y. pseudotuberculosis* (12). If, because of this difference, NghA is more active on *Y. pseudotuberculosis* than on *Y. pestis* ECM, higher levels of NghA would then be needed to observe an effect on *Y. pestis* biofilm accumulation. Thus, a fitness advantage of *nghA* mutation may have been greater in the progenitor strain. Nonetheless, fixation of alleles within populations can be driven by even slight selective advantages, especially within small effective populations of bacteria adapting to novel environments (4, 26, 34). Furthermore, the transmission efficiency and vector competence of fleas for *Y. pestis* is low, even for *X. cheopis*, considered to be the most proficient plague vector because it becomes blocked more frequently than other flea species (1, 35). Thus, at the ecological level, even a small augmentative effect on flea-borne transmission due to a loss of NghA activity may have been sufficient to confer a selective advantage to the *Y. pestis* progenitor strain. Previously identified steps in the evolutionary transition of *Y. pestis* from a food- and waterborne pathogen to an arthropod-borne pathogen include acquisition of two *Y. pestis*-unique plasmids that encode a phospholipase D required for bacterial survival and replication in the flea midgut and a plasminogen activator that enhanced invasion from an intradermal flea bite site (24, 42), as well as a reduction in toxicity toward the flea vector (17). By increasing the stability of the *Y. pestis* biofilm ECM in the flea, the loss of glycosyl hydrolase activity may have been an incremental step in the evolution of vector-borne transmission. Thus, like the recently described gene *rcsA* (46a), *nghA* may represent an antitransmission factor, analogous to antivirulence genes that enhance bacterial fitness in mammalian hosts (38), and its inactivation in *Y. pestis* may be an example of selective gene loss that contributed to the emergence of flea-borne transmission.

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