

Identification of *gmhA*, a *Yersinia pestis* Gene Required for Flea Blockage, by Using a *Caenorhabditis elegans* Biofilm System

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***Yersinia pestis*, the cause of bubonic plague, blocks feeding by its vector, the flea. Recent evidence indicates that blockage is mediated by an in vivo biofilm. *Y. pestis* and the closely related *Yersinia pseudotuberculosis* also make biofilms on the cuticle of the nematode *Caenorhabditis elegans*, which block this laboratory animal's feeding. Random screening of *Y. pseudotuberculosis* transposon insertion mutants with a *C. elegans* biofilm assay identified *gmhA* as a gene required for normal biofilms. *gmhA* encodes phosphoheptose isomerase, an enzyme required for synthesis of heptose, a conserved component of lipopolysaccharide and lipooligosaccharide. A *Y. pestis gmhA* mutant was constructed and was severely defective for *C. elegans* biofilm formation and for flea blockage but only moderately defective in an in vitro biofilm assay. These results validate use of the *C. elegans* biofilm system to identify genes and pathways involved in *Y. pestis* flea blockage.**

Yersinia pestis, the causative agent of bubonic plague, is transmitted to mammals primarily by the bites of infected fleas. The bacteria heavily colonize the insect's proventriculus, a valve-like organ connecting the esophagus to the midgut, and thereby block the passage of food. As fleas starve they bite repeatedly in search of blood meals, thus spreading *Y. pestis* to new hosts.

Y. pestis cells in the blocked proventriculus are surrounded by extracellular material that is positive for polysaccharide in histological tests (12). *Y. pestis* lacking the *hmsHFRS* operon, which encodes predicted polysaccharide biosynthetic proteins, fails to block fleas (10). *Y. pestis* flea blockage therefore appears to depend on creation of an in vivo biofilm, i.e., a population of bacteria in a self-synthesized matrix containing exopolysaccharide (EPS).

Y. pestis and its recent ancestor, *Yersinia pseudotuberculosis* (1), make biofilms on the head of the model nematode *Caenorhabditis elegans* that cover the mouth and block the worm from feeding on bacteria (4, 13). Like flea blockage, this process requires *hmsHFRS*, suggesting that the nematode can be used as a flea surrogate to study plague transmission (4). Lectin staining confirms that the matrix on nematodes contains polysaccharide (21).

The outer membranes of gram-negative bacteria contain either lipopolysaccharide (LPS) or lipooligosaccharide (LOS). *Y. pseudotuberculosis* makes a typical LPS, which comprises three covalently linked domains. Lipid A is innermost; core oligosaccharide, a branched, nonrepeating unit, is linked to lipid A; and O antigen, a branched polysaccharide containing many repeated units, is linked to core. *Y. pestis* makes only LOS, which contains lipid A and core oligosaccharide but lacks O antigen (17).

Core oligosaccharide is divided into inner and outer do-

main. Inner core contains 3-deoxy-D-manno-octulosonic acid, of which one residue is linked to lipid A, and L-glycero-D-manno heptose (heptose), of which one residue is attached to 3-deoxy-D-manno-octulosonic acid. This arrangement is common to enteric bacteria (18), including both *Y. pestis* and *Y. pseudotuberculosis* (24). Heptose is the attachment point for the more variable outer-core sugars, which in turn are the attachment sites for O antigen in organisms that make complete LPS.

Phosphoheptose isomerase, encoded by *gmhA* (previously named *lpcA*), catalyzes conversion of sedoheptulose 7-phosphate to D-glycero-D-mannoheptose 7-phosphate, the first step in the heptose biosynthesis pathway (3). *Escherichia coli* strains with mutations in *gmhA* do not make heptose, and their LPS is truncated (3) because the attachment point for more distal moieties is absent. We show that *Y. pestis* and *Y. pseudotuberculosis gmhA* mutants have truncated LOS and LPS and makes small, aberrant biofilms on *C. elegans*. The *Y. pestis gmhA* mutant is almost completely defective in blocking the flea proventriculus.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains and plasmids are described in Table 1. Bacteria were grown on L agar plates or in LB broth for genetic manipulation and strain construction. The antibiotics used and their concentrations for both *Yersinia* spp. and *E. coli* were ampicillin (100 to 200 µg/ml), chloramphenicol (10 µg/ml), kanamycin (30 µg/ml), and streptomycin (100 µg/ml).

For biofilm production, bacteria were grown overnight at 26°C in LB with shaking. Aliquots of 100 µl were pipetted onto 6-cm-diameter nematode growth medium (NGM) plates (25) that contained 2% agar. Lawns were grown 16 to 24 h at room temperature before addition of nematodes. For lawns containing mixtures of wild-type and *hms* mutant strains, each strain was grown overnight in LB, the optical density at 600 nm determined, and the cell densities adjusted with LB to make them equivalent. Mixtures at various ratios were made and immediately used to inoculate NGM plates.

Biofilm formation on nematodes. Gravid *C. elegans* adult hermaphrodites (wild-type strain N2) were placed on bacterial lawns, allowed to lay eggs for 2 to 4 h, and then removed. The plates were incubated at 20 to 22°C for 2 days, and the developmental stage of the broods was scored. In every experiment a parallel assay was conducted with either *E. coli* OP50, the standard *C. elegans* food

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype and/or description	Reference or source
<i>E. coli</i>		
DH10B	Cloning strain	Invitrogen
HB101	Cloning strain	19
OP50	Standard <i>C. elegans</i> food	25
SM10 λ pir	Conjugation donor	20
<i>Y. pestis</i>		
KIM6+	Wild type	16
CDY113	Δ pgm	4
CDY140	Δ gmhA::cat	This study
CDY145	Δ gmhA::cat/pCBD32	This study
CDY146	Δ gmhA::cat/pCR2.1-TOPO	This study
<i>Y. pseudotuberculosis</i>		
YPIII	Wild type	8
CDY103	<i>hmsT</i> ::TnphoA	4
CDY139	Δ gmhA::cat	This study
CDY143	Δ gmhA::cat/pCBD32	This study
CDY144	Δ gmhA::cat/pCR2.1-TOPO	This study
JM427	<i>dhcC</i> ::mini-Tn5	14
JM496	<i>wzc</i> ::mini-Tn5	14
JM543	<i>gmd</i> ::mini-Tn5	14
Plasmids		
pCR2.1-TOPO	Cloning vector for PCR products	Invitrogen
pCBD32	<i>gmhA</i> in pCR2.1-TOPO	This study
pCBD41	<i>gmhA</i> allelic exchange plasmid	This study
pCVD442	Allelic exchange vector	6
pRT733	Suicide plasmid for TnphoA delivery	22
pRY109	Source of <i>cat</i> gene	26

source, or *E. coli* HB101, a laboratory cloning strain that also supports nematode growth. Triplicate samples were used in each experiment.

Transposon mutagenesis of *Y. pseudotuberculosis* JM403, a spontaneous streptomycin-resistant variant of *Y. pseudotuberculosis* YPIII (gift of J. Mecsas), has no defect in nematode growth inhibition (data not shown). Plasmid pRT733, which contains transposon TnphoA and which does not replicate in *Y. pseudotuberculosis*, was delivered into JM403 by conjugation from *E. coli* strain SM10 λ pir essentially as described previously (22). *Y. pseudotuberculosis* containing the transposon was selected on L agar with kanamycin and the *E. coli* donors counterselected with streptomycin. Colonies were tested individually in the nematode growth assay, and mutants were kept either if they formed no biofilm on nematodes or if they formed a biofilm but the animals nevertheless grew more rapidly than on JM403. Although the alkaline phosphatase encoded by TnphoA can be used to identify genes whose products may be exported, screening was without regard to export phenotypes.

Transposon insertion mapping. Chromosomal DNA was digested with BamHI and SalI, neither of which cuts in the kanamycin resistance gene of TnphoA. Fragments were ligated into double-digested pUC18 and transformed into *E. coli* with selection for kanamycin resistance. The sequence of *Y. pseudo-*

tuberculosis DNA flanking the transposon was determined using a primer that hybridizes to the 5' end of TnphoA (Table 2).

Cloning and sequencing of *Y. pseudotuberculosis gmhA*. Using the genome sequence of *Y. pestis* CO92 (15), PCR primers were designed to amplify chromosomal DNA of *gmhA*, including all 221 nucleotides intervening between *gmhA* and the upstream open reading frame *yafH* (Table 2). Two independent PCR products from YPIII template DNA were sequenced, without cloning, using the same primers. The PCR product was cloned into the vector pCR2.1-TOPO, using *E. coli* DH10B as host, to create pCBD32.

Construction of *gmhA* deletion mutants. Approximately 900 bp of DNA on each side of *gmhA* was amplified by PCR using the primers shown in Table 2. The products were digested with the enzymes indicated and then ligated into the allelic replacement vector pCVD442 along with a PstI fragment containing the *cat* (chloramphenicol resistance) gene of pRY109. The resulting plasmid, pCBD41, was transformed into the *E. coli* conjugation donor strain SM10 λ pir (20), which was then mated to *Y. pseudotuberculosis* YPIII and *Y. pestis* KIM6+. Exconjugants were selected on *Yersinia* selective agar (Difco) containing chloramphenicol and ampicillin. Plasmid replication requires the *pir* gene, which is absent in *Yersinia* spp., and therefore the selection recovered presumed plasmid cointegrates at the *gmhA* chromosomal locus. To obtain a second recombination that removed *gmhA* and plasmid sequences, cells were plated on L agar containing chloramphenicol and 5% sucrose but lacking NaCl; this selected against cells retaining the vector-carried *sacB* gene, which is lethal in the presence of sucrose. After confirmation of the loss of ampicillin resistance, deletion of *gmhA* and replacement with *cat* were confirmed by PCR assays and Southern hybridization (data not shown).

Analysis of LPS and LOS. Bacteria were grown on NGM agar. Cells were lysed in a buffer of 0.63 M Tris-HCl, pH 6.8, 1% sodium dodecyl sulfate, 10% sucrose, 0.008 M tetrasodium-EDTA, and 0.004% bromophenol blue. After boiling for 5 min, proteinase K was added to 1.5 mg/ml and the samples incubated for 2 to 3 h at 60°C. Samples were boiled again and immediately electrophoresed in gels containing 15% acrylamide, 0.2% sodium dodecyl sulfate, and 4 M urea. Gels were fixed and silver stained as described previously (23).

Flea infections. *Xenopsylla cheopis* fleas from colonies maintained at the Rocky Mountain Laboratories were infected by using an artificial feeding system (10). Bacteria were grown in brain heart infusion agar at 37°C without aeration for 16 h, quantified by direct counting in a Petroff-Hausser counting chamber, recovered by centrifugation, and resuspended in phosphate-buffered saline at $\sim 2.5 \times 10^9$ /ml. The infectious blood meal was prepared by adding 1 ml of the bacterial suspension to 5 ml of fresh heparinized mouse blood. Fleas that took a blood meal after a 1-h feeding period were kept at 21°C and 75% relative humidity and fed twice weekly on uninfected mice. One sample of 50 male and 50 female fleas was monitored for 4 weeks for mortality and proventricular blockage as described previously (10, 12). Additional samples of 20 female fleas were placed at -80°C immediately after the infectious blood meal and after 7 and 28 days to determine the infection rate and bacterial load per flea. Thawed fleas were surface sterilized and individually triturated in 50 μ l brain heart infusion and 20 μ l glass sand; dilutions of the triturates were plated on *Yersinia* selective agar (8, 10). *Y. pestis* CFU were counted after 48 h of incubation at 28°C.

Growth and detection of biofilms in polystyrene dishes. Bacteria were inoculated in 1 ml of NGM broth and incubated in 24-well polystyrene culture plates at 25°C for 4 days. Broth-only samples were the negative control. Cultures were removed, and the wells were washed three times with wheat germ agglutinin (WGA) buffer (0.01 M phosphate, 0.15 M NaCl, pH 7.3). WGA conjugated to alkaline phosphatase (EY Laboratories) was added to each well at 1 μ g/ml in WGA buffer and incubated at room temperature for 90 min, after which the wells

TABLE 2. PCR and sequencing primers

Primer	Sequence ^a	Purpose
TNPHOAS	5'-TACTTGTGTATAAGAGTCAG	Sequence out from TnphoA 5' end
GMHF	5'-GCTTGGATCCCATAATGAAGCTCCTGAGATGTAG	PCR, complete <i>gmhA</i> locus
GMHR	5'-AGTGGGTCGACACAGAAGATTGAGGTGATCAAC	PCR, complete <i>gmhA</i> locus
GMHUPF	5'-GTTCTAGAGCATCAGAACCAGCTTCC	PCR, upstream flanking DNA
GMHUPR	5'-TCCTGCAGTTTTCTCAATCAGTGTACCG	PCR, upstream flanking DNA
GMHDNF	5'-TCCTGCAGCCTCAATCTTCTGTGAACATT	PCR, downstream flanking DNA
GMHDNR	5'-CCGCATGCGCACTAATTAACCGACCCG	PCR, downstream flanking DNA

^a Underlined bases are restriction sites designed for cloning; sites in GMHF and GMHR ultimately were not used. Sites in other primers are as follows: GMHUPF, XbaI; GMHUPR and GMHDNF, PstI; GMHDNR, SphI.

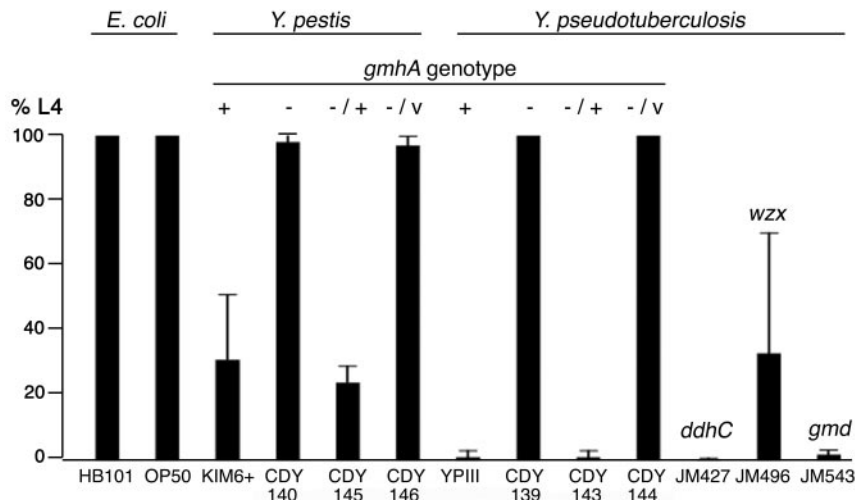


FIG. 1. Nematode growth on *Y. pestis* and *Y. pseudotuberculosis* strains. The percentage of L4 *C. elegans* was determined 2 days after eggs were laid on lawns of the indicated strains. *gmhA* genotypes: +, wild type; -, chromosomal deletion; -/+, deletion strain carrying complementing plasmid pCBD32; -/v, deletion strain carrying vector. Data are means and standard deviations from three independent experiments for each strain except YPIII, which was assayed six times.

were washed three times with buffer alone. Substrate stock solution (nitroblue tetrazolium chloride [18.75 mg/ml] and 5-bromo-4-chloro-3-indolyl phosphate [9.4 mg/ml] in 67% dimethyl sulfoxide) was diluted 1:50 just before use into staining buffer (0.1 M Tris, 0.05 M MgCl₂, 0.1 M NaCl, pH 9.5). One milliliter of staining solution was added to each well and incubated at room temperature for 3 to 4 h. Wells were washed three times with deionized water, 1 ml of ethanol was added, and plates were covered and wrapped with paraffin film to prevent evaporation. Plates were incubated overnight at room temperature with gentle rotation (60 rpm) to dissolve the stained material, after which absorbance was measured at 425 nm. Each strain was tested in quadruplicate.

RESULTS

Screen for biofilm-defective *Y. pseudotuberculosis*. *C. elegans* development is highly synchronous when the nematodes are fed nonpathogenic *E. coli* strains. At 20 to 22°C, all animals reach the fourth larval stage of development (L4) 2 days after eggs are laid (Fig. 1). In contrast, the biofilm produced by *Y. pseudotuberculosis* YPIII covers the nematode mouth and inhibits feeding (Fig. 2), such that almost no animals are L4 after 2 days (Fig. 1). Under the same conditions, *Y. pestis* KIM6+ inhibits growth only partially, which would complicate identification of defective bacteria in a genetic screen. We therefore employed a two-step strategy to identify *Y. pestis* biofilm-related genes. First, we generated transposon insertions in *Y. pseudotuberculosis* and screened for mutants that permit normal nematode growth, indicating a biofilm defect. Next, we constructed mutations in the *Y. pestis* homologues of the identified genes and tested for defects in biofilm formation on *C. elegans* and in flea blockage.

Y. pseudotuberculosis was mutagenized with transposon TnphoA. Of approximately 1,400 mutant colonies tested in the *C. elegans* growth assay, 4 were confirmed defective. One, with a mutation in *hmsT*, has been described previously (4), and two others have not yet been characterized. Analysis of the fourth mutant by Southern hybridization indicated that two copies of TnphoA were inserted in the chromosome (data not shown). DNA flanking one insertion was cloned and sequenced, pro-

ducing a match to *Y. pestis gmhA*. Because a *Y. pseudotuberculosis* genomic sequence was not yet available, we took advantage of the high degree of sequence similarity between *Y. pestis* and *Y. pseudotuberculosis*. The genome sequence of *Y. pestis* was used to design PCR primers to amplify the *gmhA* locus, including the complete coding region and an additional 221 base pairs extending 5' to the next open reading frame upstream. The double TnphoA mutant was transformed with the cloned PCR product from *Y. pseudotuberculosis*, and this restored *C. elegans* growth inhibition (data not shown). The second transposon insertion was not examined further.

***gmhA* is required for normal biofilm formation by both *Y. pestis* and *Y. pseudotuberculosis*.** Strains with complete *gmhA* deletions were constructed in both *Y. pseudotuberculosis* YPIII and *Y. pestis* KIM6+ backgrounds and assayed for nematode growth inhibition (Fig. 1). The *Y. pestis* mutant, although strongly defective, consistently inhibited growth of about 2% of nematodes. The *Y. pseudotuberculosis* mutant was completely defective, permitting 100% of animals to reach stage L4. Transformation with the plasmid-carried wild-type *Y. pseudotuberculosis gmhA* complemented the defect in both species. The growth rate of each mutant was the same as that of the corresponding wild type (data not shown).

Nematodes were incubated on bacterial lawns overnight, washed to remove loosely adhering material, and examined by differential interference contrast microscopy (Fig. 2). Wild-type bacteria of both species produced copious biofilms that covered the mouths of the animals and also adhered to their sides. *gmhA* mutants of both species made small biofilms, but their appearances differed. The *Y. pestis* mutant biofilms adhered directly to the mouths of some worms, consistent with its low level of growth inhibition. The *Y. pseudotuberculosis gmhA* biofilms adhered to the side of the head but never covered the mouth, so feeding was not impaired.

The failure of mutant biofilms to bind the mouth could result from a change in the amount of EPS, its composition, or

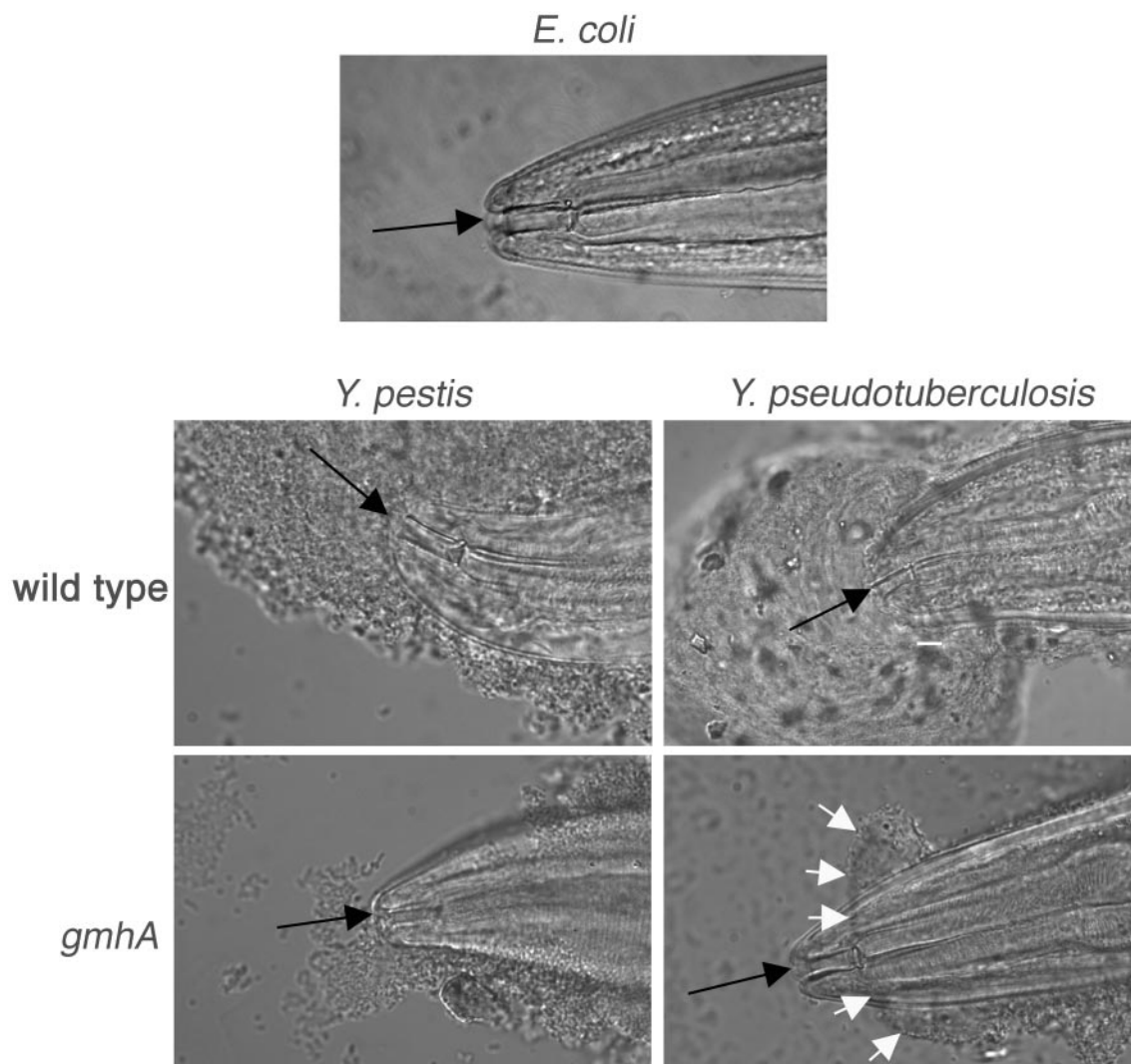


FIG. 2. Biofilms on *C. elegans* made by *Yersinia* wild-type and *gmhA* mutant strains. A nematode grown on *E. coli* food strain OP50 is shown for comparison. Black arrows, mouth. Wild-type *Y. pestis* and *Y. pseudotuberculosis* biofilms cover the mouth completely and block feeding. Most *Y. pestis gmhA* biofilms fail to attach to the mouth (not shown), but a few are as shown, accounting for the 2% of *C. elegans* animals that fail to grow normally on this strain (Fig. 1). *Y. pseudotuberculosis gmhA* biofilms attach to the side of the head posterior to the mouth; white arrows mark the forward edge of the biofilm.

both. To examine the role of EPS quantity, *Yersinia* lawns were made using various ratios of wild-type bacteria to *hms* mutants, which have defects in a polysaccharide synthesis pathway and which make no biofilms on *C. elegans* (4). *Y. pestis* KIM6+ wild type was mixed with CDY113, which contains a spontaneous deletion of the 102-kb *pgm* locus that includes *hmsHRFS* (7). *Y. pseudotuberculosis* YPIII wild type was mixed with CDY103, a biofilm-defective strain with a mutation in *hmsT* (4). For *Y. pestis*, a 0.17 ratio of wild-type to *pgm* inoculum produced small biofilms that failed to cover the mouths of the worms and allowed more than 98% of nematodes to grow normally, essentially reproducing the *gmhA* phenotype. The *Y. pseudotuberculosis gmhA* phenotypes also could be reproduced by this method, although greater dilution was required (data not shown). Although the possibility that the *gmhA* biofilms have an altered composition cannot be excluded, the phenotypes are explainable solely by diminished production of EPS.

The *gmhA* open reading frame of *Y. pseudotuberculosis* strain YPIII was sequenced and found to be identical to that of *Y. pestis* strain KIM (5), the parent strain of KIM6+. During the course of this work the genomic sequence of *Y. pseudotuberculosis* strain IP32953 was determined, and it contained the identical *gmhA* coding sequence (GenBank accession no. NC 006155). *gmhA* of *Y. pestis* CO92 differs in one nucleotide from both *Y. pseudotuberculosis* and *Y. pestis* KIM, giving CO92 an aspartic acid at residue 109 instead of glycine. The KIM and *Y. pseudotuberculosis* polypeptides of 193 amino acids are 91% identical to that of the *E. coli* protein.

gmhA encodes phosphoheptose isomerase, an enzyme in the biosynthetic pathway of heptose (3). Mutations that abrogate heptose synthesis cause truncation of LPS or LOS (3). We examined *Y. pseudotuberculosis* LPS and *Y. pestis* LOS on silver-stained gels after electrophoresis. In both species, the core of the *gmhA* mutant migrated faster than that of the wild type,

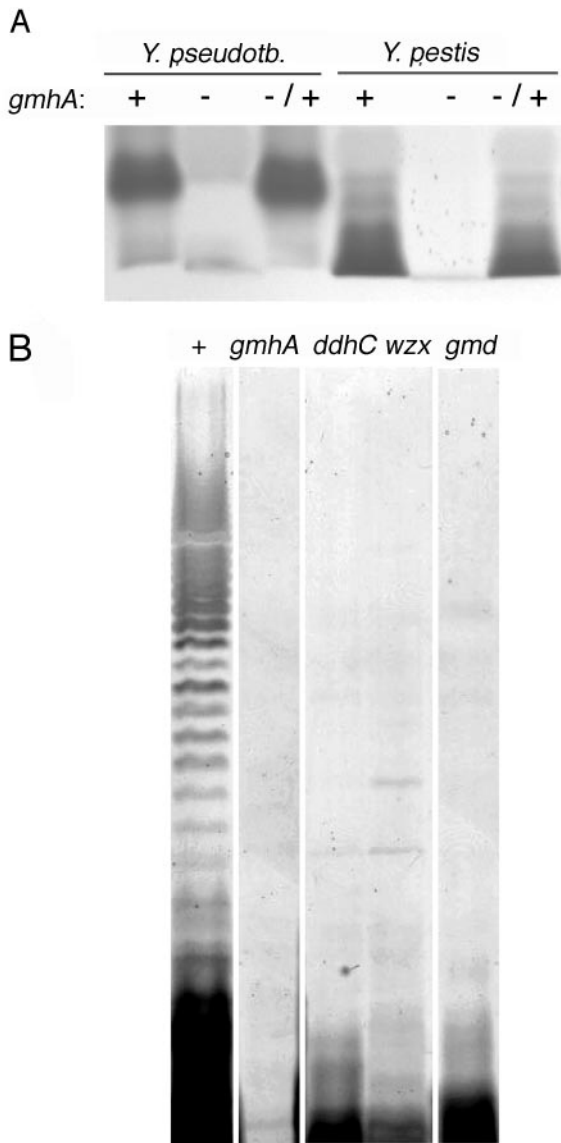


FIG. 3. LOS and LPS of *Yersinia* mutants. (A) Core oligosaccharide of *gmhA* mutants migrates faster through gels, indicating truncation. +, wild type; -, *gmhA* deletion mutant; -/+, mutant complemented with pCBD32. (B) O antigen is absent in the *Y. pseudotuberculosis gmhA* mutant and in strains with mutations in known O-antigen genes. Wild-type YPIII (+) shows a typical ladder-like pattern produced by variable numbers of O-antigen repeat units. Faint bands in mutant samples are presumed protease-resistant proteins.

indicating that it was truncated (Fig. 3A). Also as expected, O antigen was absent in the *Y. pseudotuberculosis gmhA* mutant (Fig. 3B).

O antigen is not required for *Y. pseudotuberculosis* biofilms. Wild-type *Y. pestis* lacks O antigen (17) yet makes biofilms on *C. elegans*. It therefore seemed probable that *Y. pseudotuberculosis* biofilms would not require O antigen. We confirmed this by examining *Y. pseudotuberculosis* strains with transposon insertions in O-antigen biosynthetic genes *ddhC*, *wzx*, and *gmd* (14). The absence of O antigen in each was confirmed by silver

staining (Fig. 3B). When tested in the nematode growth assay, the *ddhC* and *gmd* mutants were indistinguishable from the wild type (Fig. 1), indicating that O antigen is not required for biofilm formation. The *wzx* mutant made a visible biofilm on *C. elegans* (data not shown) but for unknown reasons was highly variable in nematode growth assays (Fig. 1).

The *Y. pestis gmhA* mutant is defective for flea blockage. The *Y. pestis gmhA* mutant CDY140 was tested for flea blockage in parallel with its parent strain, KIM6+. The mutant was able to colonize fleas (Fig. 4A), but proventriculus blockage was almost entirely absent (Fig. 4B). The mutant grew in fleas to the same titer as the wild type (Fig. 4C), indicating that the failure to block fleas was not the result of a growth defect in vivo. These results indicate that *gmhA* plays a specific role in blockage, rather than a general role in colonization. The *C. elegans* results described above suggest that the defect in fleas is due to reduced EPS production.

Lack of correlation between biofilms in vivo and in vitro. Biofilms are frequently studied in vitro by growing them on solid artificial surfaces such as glass and plastic. We examined *Yersinia* wild-type and *gmhA* mutants by growing standing broth cultures in polystyrene dishes, using the same medium (NGM) as for *C. elegans* biofilms except without agar (Fig. 5). *Y. pseudotuberculosis* YPIII made a substantial biofilm, and its isogenic *gmhA* mutant was severely defective ($P = 0.001$, two-tailed Student *t* test). *Y. pestis* KIM6+ made a smaller biofilm than YPIII, consistent with its lesser ability to inhibit *C. elegans* growth on NGM agar. However, the isogenic *Y. pestis gmhA* made only slightly less biofilm than its parent. In repeated trials the mutant always made less than the wild type, but the difference was not statistically significant (e.g., $P = 0.08$ for Fig. 5). This is in contrast to the almost complete failure of *Y. pestis gmhA* to either inhibit *C. elegans* growth (Fig. 1) or block fleas (Fig. 4).

DISCUSSION

Recent evidence indicates that *Y. pestis* flea blockage is mediated by a biofilm the bacteria produce in the insect proventriculus. Microscopic examination shows bacteria embedded in an extracellular matrix that stains positively for polysaccharide (12). Blockage requires the *Y. pestis hmsHFRS* operon (10), which is also required to make biofilms on *C. elegans* (4). Although polysaccharide synthesis by *hmsHFRS* gene products has not been demonstrated directly, HmsR contains a conserved glycosyltransferase domain, Pfam 00535 (2). A protein containing this domain, *Staphylococcus epidermidis* IcaA, is required for biofilm formation and for in vitro synthesis of a homopolymer of $\beta(1,6)$ -linked *N*-acetyl-D-glucosamine (9).

Screening for defects in *C. elegans* biofilms identified *gmhA*, and we showed that this gene is also required for *Y. pestis* flea blockage. Biochemical evidence from *E. coli* indicates that GmhA is a phosphoheptose isomerase. *E. coli* cells lacking this enzyme cannot synthesize heptose and have severely truncated LPS (3). The *Yersinia* GmhA amino acid sequence is 91% identical to that of *E. coli*, implying identical function. As expected from the known structures of LPS and LOS, mutations in *gmhA* increased the electrophoretic mobility of core oligosaccharide components for both *Yersinia* species and eliminated O antigen from *Y. pseudotuberculosis* (Fig. 3).

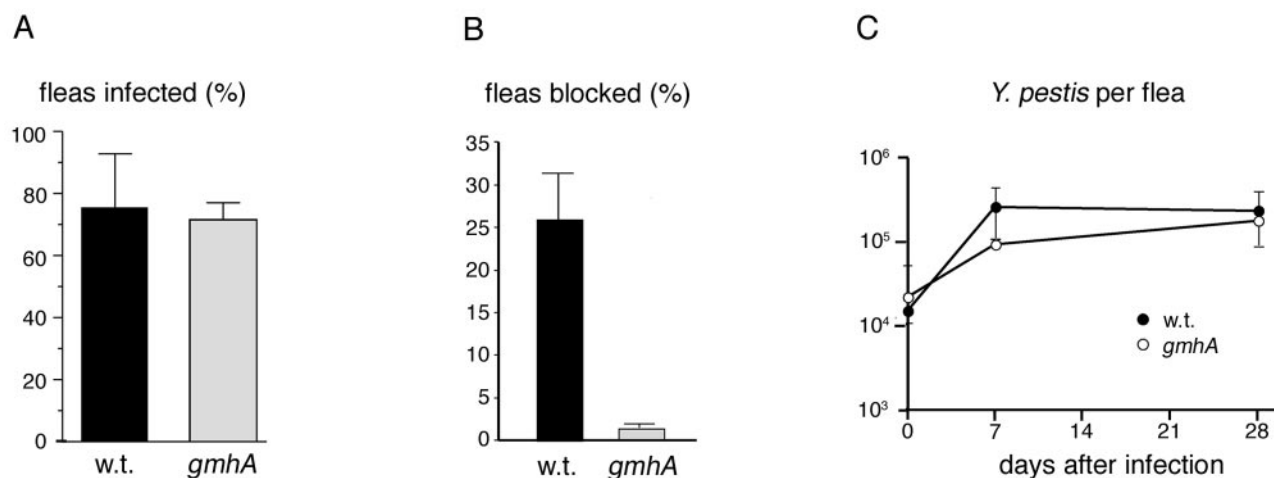


FIG. 4. The *Y. pestis gmhA* mutant is defective for flea blockage. (A) Colonization of flea digestive tract after a single blood meal containing *Y. pestis*. (B) Flea blockage during the 4-week period after an infected blood meal. The 25% level of blockage for the wild type is typical for strain KIM6+. (C) *Y. pestis* CFU taken up in the infectious blood meal (day 0) and in infected fleas 7 and 28 days postinfection. Data are means and standard deviations from two independent experiments. w.t., wild type.

Although *gmhA* mutants make visible biofilms on *C. elegans*, these biofilms have scant ability to block nematode feeding (Fig. 1) and are less substantial than those made by the wild type (Fig. 2). In the case of *Y. pseudotuberculosis*, the aberrant biofilms were never observed to cover the mouth. With *Y. pestis* the biofilms attached to the mouths of some animals (Fig. 2), but only rarely was this sufficient to block feeding and inhibit growth. The presence of a visible biofilm indicates that the defect cannot be a complete loss of EPS. By titrating wild-type bacteria with *hms* mutants that are biofilm defective, so that the mixed cultures made less EPS, we reproduced the phenomenon of small biofilms that attach to the side of the *C. elegans* head but not to the mouth. This suggests that the defect in *gmhA* mutants is because of diminished EPS production rather

than a change in the EPS composition or structure. These observations, incidentally, suggest that the surface composition of the *C. elegans* cuticle differs between the mouth and the side of the head nearby and that the EPS has greater affinity for the latter.

Heptose apparently is not a constituent of the normal biofilm, since none was detected by gas chromatography and mass spectrometry of *Y. pestis* EPS purified from biofilms on nematode (J. Cipollo, personal communication). The biofilm phenotypes of *gmhA* mutants therefore appear to be an indirect effect, most likely from truncation of the core oligosaccharide of LOS and LPS. An indirect role for core heptose was suggested by a study of biofilm formation in vitro by *Klebsiella pneumoniae* and *Proteus mirabilis*. Mutations in *waaE*, which encodes a protein involved in adding a substitution on inner-core heptose, resulted in 40% reductions in biofilm (11). A possible explanation for this defect is that a component of the biofilm synthesis and export machinery interacts with the substituted heptose in the core. Similarly, it may be that in wild-type *Yersinia* spp., outer membrane proteins involved in EPS synthesis, processing, or export interact with core heptose or with a distal core component.

The *Y. pestis gmhA* mutant is severely defective for flea blockage (Fig. 4B). This defect is not due to an inability to colonize fleas, because the mutant colonized as many fleas as did the wild type (Fig. 4A) and grew in the insect to the same extent (Fig. 4C). Rather, the defect specifically involves proventriculus blockage. Significantly, a small fraction of fleas were blocked (Fig. 4B), just as a small fraction of *C. elegans* animals were inhibited from growth by *gmhA* mutant biofilms (Fig. 1). This suggests that in the flea, as on the nematode, the *gmhA* mutant is able to produce a small amount of EPS. These results also indicate the *C. elegans* biofilm closely mimics some aspects of flea blockage, further validating the nematode model.

Screening *Y. pestis* mutants directly in fleas to identify genes involved in blockage is impractical due to the slow growth of the insects, the need to dissect each flea, and the fact that only

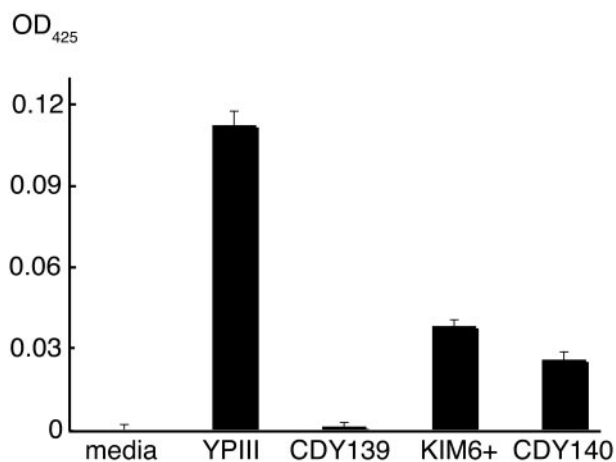


FIG. 5. In vitro biofilms. Bacteria were grown standing for 4 days in polystyrene dishes, after which broth and planktonic cells were removed and adherent biofilm matrix was detected with WGA conjugated to alkaline phosphatase (see Materials and Methods for details). Data are means and standard errors of the means for four replicates in a single experiment; results are typical of multiple experiments. OD₄₂₅, optical density at 425 nm.

a fraction of infected fleas become blocked. On the other hand, screening for biofilm-defective *Yersinia* spp. by using *C. elegans* is efficient because the nematodes grow rapidly in a simple culture system, the biofilm is visible on the exterior of intact worms, and close to 100% of animals are affected when the biofilm is produced by *Y. pseudotuberculosis*.

Our results with *in vitro* biofilms (Fig. 5) suggest that biofilms grown on polystyrene are inferior to those produced on *C. elegans* as a model for the *in vivo* flea biofilm. When grown on polystyrene, the *Y. pestis gmhA* mutant has only a slight defect that does not rise to the level of statistical significance. Screening in this *in vitro* system therefore could fail to recover informative mutants. In contrast, identification of *gmhA* confirms that using the *C. elegans* biofilm assay is a practical means to find additional *Y. pestis* genes with roles in flea blockage.

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