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Host-Microbe Biology

Differential Gene Expression Patterns of *Yersinia pestis* and *Yersinia pseudotuberculosis* during Infection and Biofilm Formation in the Flea Digestive Tract

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ABSTRACT *Yersinia pestis*, the etiologic agent of plague, emerged as a fleaborne pathogen only within the last 6,000 years. Just five simple genetic changes in the *Yersinia pseudotuberculosis* progenitor, which served to eliminate toxicity to fleas and to enhance survival and biofilm formation in the flea digestive tract, were key to the transition to the arthropodborne transmission route. To gain a deeper understanding of the genetic basis for the development of a transmissible biofilm infection in the flea foregut, we evaluated additional gene differences and performed *in vivo* transcriptional profiling of *Y. pestis*, a *Y. pseudotuberculosis* wild-type strain (unable to form biofilm in the flea foregut), and a *Y. pseudotuberculosis* mutant strain (able to produce foregut-blocking biofilm in fleas) recovered from fleas 1 day and 14 days after an infectious blood meal. Surprisingly, the *Y. pseudotuberculosis* mutations that increased c-di-GMP levels and enabled biofilm development in the flea did not change the expression levels of the *hms* genes responsible for the synthesis and export of the extracellular polysaccharide matrix required for mature biofilm formation. The *Y. pseudotuberculosis* mutant uniquely expressed much higher levels of *Yersinia* type VI secretion system 4 (T6SS-4) in the flea, and this locus was required for flea blockage by *Y. pseudotuberculosis* but not for blockage by *Y. pestis*. Significant differences between the two species in expression of several metabolism genes, the Psa fimbrial genes, quorum sensing-related genes, transcription regulation genes, and stress response genes were evident during flea infection.

IMPORTANCE *Y. pestis* emerged as a highly virulent, arthropod-transmitted pathogen on the basis of relatively few and discrete genetic changes from *Y. pseudotuberculosis*. Parallel comparisons of the *in vitro* and *in vivo* transcriptomes of *Y. pestis* and two *Y. pseudotuberculosis* variants that produce a nontransmissible infection and a transmissible infection of the flea vector, respectively, provided insights into how *Y. pestis* has adapted to life in its flea vector and point to evolutionary changes in the regulation of metabolic and biofilm development pathways in these two closely related species.

KEYWORDS *Yersinia pestis*, *Yersinia pseudotuberculosis*, arthropodborne transmission, biofilm, flea, plague

Yersinia pestis is transmitted by fleas and is perhaps the newest arthropodborne pathogen, having adopted this transmission route only within the last 6,000 years (1–3). Its closely related recent ancestor *Yersinia pseudotuberculosis* maintains the fecal-oral transmission route via contaminated food and water in a manner that is

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typical of an enteric pathogen. Efficient transmission of *Y. pestis* during a flea bite depends on its ability to colonize the proventriculus, a valve in the foregut located between the esophagus and the midgut, in the form of bacterial aggregates associated with partially digested blood meal material. The proventricular aggregate can physically impede the ingestion of blood into the midgut and result in regurgitation of some blood mixed with bacteria (4). This transmission mechanism is greatly enhanced after the development of a mature biofilm, which is required for permanent, consolidated colonization of the proventriculus (4–7). Continued growth of the biofilm can eventually completely block the proventriculus. Biofilm maturation and permanent colonization of the proventriculus depend on the production by *Y. pestis* of poly-β-1,6-*N*-acetyl-D-glucosamine exopolysaccharide, an essential component of the extracellular matrix that holds the biofilm together and mediates its adherence to a surface, for example, to the spines that line the lumen of the proventriculus (6, 8–10). The *Yersinia hmsHFRS* operon is responsible for the synthesis and export of this exopolysaccharide (9, 11).

In keeping with a recent evolutionary leap, just five simple discrete genetic changes in the *Y. pseudotuberculosis* ancestor were key to the ability to use the flea as an efficient transmission vector (12). The phospholipase D gene *ymt*, present on one of the two plasmids acquired by *Y. pestis* since its divergence from *Y. pseudotuberculosis*, greatly enhanced survival in the flea midgut (13). Three loss-of-function mutations in genes that resulted in increased intracellular levels of c-di-GMP, an activator of bacterial biofilm development, allowed stable colonization of the flea foregut and regurgitative transmission during a flea bite. A fifth genetic change, loss of *ureD* function, eliminated the high mortality of infected fleas caused by the toxic effects of urease activity (14).

Although a *Y. pseudotuberculosis* strain with these few genetic changes is efficiently transmitted by fleas, it caused complete blockage in only ~12% of infected fleas, compared to the ~40% blockage rate of *Y. pestis* (12). The c-di-GMP metabolic genes and the *hmsHFRS* operons are identical in the two strains, indicating that additional genetic or regulatory differences between the two species that are related to biofilm development remain to be discovered. In an attempt to identify these differences, we compared the transcriptional profiles of *Y. pestis* and *Y. pseudotuberculosis* during infection of the flea and in temperature-matched *in vitro* cultures. We also examined the effects on biofilm development *in vitro* and in the flea that additional selected genetic differences between the two species have.

RESULTS AND DISCUSSION

Comparative analysis of *in vitro* and *in vivo* biofilm-related phenotypes. We previously described a genetically defined *Y. pseudotuberculosis* mutant strain that is able to produce a transmissible infection in fleas (12, 14). However, although the numbers of CFU transmitted by this *Y. pseudotuberculosis* variant (referred to here as the IPmt strain) and by *Y. pestis* were equivalent, the incidence of complete blockage of the proventriculus was less than half that seen with *Y. pestis* (Fig. 1) (12). We made additional mutations to the flea-transmissible *Y. pseudotuberculosis* strain, to match the *Y. pestis* genotype, in genes that we suspected might be related to the biofilm phenotype. The *ddhB-gmd* O-antigen gene cluster (15) was deleted from *Y. pseudotuberculosis* IPmt to generate the SY406 derivative strain (Table 1). Further changes to SY406, including (i) deletion of YPTB0871–0880 (genes unique to *Y. pseudotuberculosis* and an adhesin gene that differs between the two species), YPTB0556–0562 (an O-antigen synthesis gene and metabolic genes not present in *Y. pestis*), YPTB2490–2497 (the *htrB* lipid A acyltransferase and *mdoGH* periplasmic glucan biosynthesis genes that are not present in *Y. pestis*) (16, 17), and the *chiC* chitinase gene that is not present in *Y. pestis* and (ii) replacement of the YPTB1709 chemotaxis gene, the *flhD* motility gene, the *nghA* biofilm-disrupting glycosyl hydrolase gene (8), and the *barA* hybrid sensory histidine kinase gene with their *Y. pestis* pseudogene counterparts, generated the SY381 strain (Table 1). However, these additional genetic changes had no augmentative effect on proventricular blockage (Fig. 1).

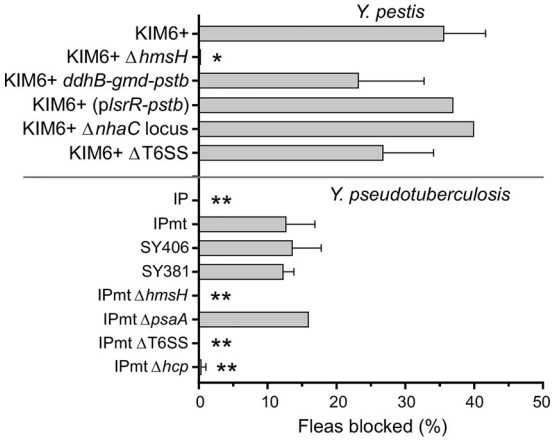

FIG 1 Percentages of *X. cheopis* fleas that developed complete proventricular blockage during the 4-week period after feeding on mouse blood containing the *Y. pestis* or *Y. pseudotuberculosis* strains indicated. The mean and standard deviation of three independent experiments that each included ~100 fleas are shown, except for the IPmt Δ*hcp* strain (2 experiments). Means without error bars indicated are the result of one experiment. Results for the IP strain are from reference 12. * and **, *P* values of <0.01 relative to *Y. pestis* KIM6+ and *Y. pseudotuberculosis* IPmt, respectively, by one-way ANOVA and Tukey's multiple-comparison test.

Y. pestis infection of the flea digestive tract is characterized by initial HmsHFRS-independent autoaggregation to form large bacterial masses that rapidly localize to the proventriculus and can eventually lead to Hms-dependent blockage (4). These *in vivo* characteristics have been correlated with autoaggregative growth in liquid media and with the formation of pigmented colonies on Congo red agar and of biofilm on plastic or glass surfaces at temperatures below 26°C, typical of the flea environment (4–6, 18, 19). We characterized these *in vitro* phenotypes for wild-type and mutant *Y. pestis* and *Y. pseudotuberculosis* strains (Fig. 2 and 3) to see how they related to the ability to block fleas (Fig. 1).

Wild-type *Y. pseudotuberculosis* colonies were only slightly pigmented on Congo red agar, whereas colonies of the flea-blocking *Y. pseudotuberculosis* strains, like wild-type *Y. pestis*, were dark red (Fig. 2). As with *Y. pestis*, the pigmentation phenotype of *Y. pseudotuberculosis* is dependent on the *hmsHFRS* genes and on temperature, with much greater pigmentation after growth at 21°C than at 37°C. *In vitro* biofilm formation results were also *hmsHFRS* dependent for both species but differed with respect to culture conditions. In LB medium, *Y. pestis* forms biofilm at 21°C but not at 37°C (6, 20); surprisingly, the *Y. pseudotuberculosis* IP and IPmt strains formed more biofilm at 37°C than at 21°C in LB (Fig. 2). In TMH, a chemically defined minimal medium, however, *Y. pseudotuberculosis* biofilm production was greater at 21°C, typical of *Y. pestis* and of the temperature at which biofilm is produced in the flea. We also noted that pigmentation of *Y. pseudotuberculosis*, but not that of *Y. pestis*, was reduced on LB agar containing Congo red compared to standard Congo red agar plates. These results suggest that production of the Hms exopolysaccharide matrix of the biofilm is differentially induced or regulated in the two species at different growth temperatures in different nutritional environments.

In contrast to the pigmentation and *in vitro* biofilm phenotypes, autoaggregation in LB medium was not dependent on *hmsHFRS*, as the biofilm- and pigmentation-negative *Y. pestis* KIM6 strain lacking this operon aggregated to the same extent as the parental KIM6+ strain (Fig. 3). Autoaggregation and sedimentation in LB medium was inhibited by production of a smooth form of lipopolysaccharide (LPS)—deletion of the O-antigen gene cluster *ddhB-gmd* (15) in *Y. pseudotuberculosis* was sufficient to induce autoaggregation (Fig. 3). Thus, autoaggregation in LB medium is not related to the rapid autoaggregation that occurs in the flea gut shortly after an infectious blood meal, because O-antigen-positive *Y. pseudotuberculosis* autoaggregates in the flea (12) but

TABLE 1 Strains and plasmids used in this study[a]

Strain or plasmid	Genotype and/or description	Reference or source
Strains		
Y. pestis CO92	Wild type (pCD1 positive; fully virulent)	93
Y. pestis KIM6+ wt	Wild type (pCD1 negative; avirulent)	94
Y. pestis KIM6 wt	Wild type (pCD1 negative; Pgm negative [*hmsHFRS* negative]; biofilm negative)	94
Y. pestis KIM6+ Δ*hmsH*	Pgm negative; biofilm negative	This study
Y. pestis KIM6+ Δ*ddhB-gmd*	Deleted of the *ddhB-gmd* region (y1069–y1079) of the O-antigen operon	This study
Y. pestis KIM6+ *ddhD-gmd-pstb*	Pseudogenized O-antigen locus replaced by functional locus of *Y. pseudotuberculosis*; makes smooth form of LPS	This study
Y. pestis KIM6+ PDE4-*pstb*	KIM6+ (pCR::YPTB3828); addition of phosphodiesterase gene on high-copy-number plasmid	This study
Y. pestis KIM6+ (p*lsrR-pstb*)	KIM6+ (pCR::*lsrR-pstb*); addition of functional LsrR repressor (YPTB0553)	This study
Y. pestis KIM6+ Δ*nhaC* locus	KIM6+ deleted of YPTB3427–3432 homologs	This study
Y. pestis KIM6+ ΔT6SS	T6SS locus 4 (y3657–y3677) deleted and replaced with a Km resistance gene	This study
Y. pestis KIM6+ Δ*hcp*	T6SS locus 4 *hcp* gene (y3673) deleted and replaced with a Km resistance gene	This study
Y. pestis KIM6+ Δy3677	T6SS locus 4 y3673 gene (YPTB0639 homolog) deleted and replaced with a Km resistance gene	This study
Y. pseudotuberculosis IP32953 IPwt	IP32953 wild type	95
Y. pseudotuberculosis IP32953 IP	IP32953 transformed with pCH16 (*ymt* on plasmid)	12
Y. pseudotuberculosis IP32953 IPmt	IP32953 *rcsA-pe* PDE2-*pe* PDE3-*pe* (pCH16) (IP with native *rcsA*, PDE2, and PDE3 genes replaced by *Y. pestis* nonfunctional alleles)	12
Y. pseudotuberculosis IP32953 SY406	IPmt deleted of *ddhB-gmd* genes (O-antigen-negative mutant)	15; this study
Y. pseudotuberculosis IP32953 SY381	IP32953 *rcsA-pe* PDE2-*pe* PDE3-*pe*YPTB1709-*pe*, Δ*ddhB gmd flhD-pe* Δ*chiC nghA-pe barA-pe* ΔYPTB2490–2497 ΔYPTB0556–0562 ΔYPTB0871-0880 ΔYPTB1894-1891::*ymt*	12; this study
Y. pseudotuberculosis IP32953 IPmt Δ*hmsH*	IPmt Pgm negative	This study
Y. pseudotuberculosis IP32953 IPmt ΔPDE4	IPmt deleted of phospodiesterase gene YPTB3828	This study
Y. pseudotuberculosis IP32953 IP ΔT6SS	T6SS locus 4 (YPTB0639–YPTB0657) deleted and replaced with a Km resistance gene	This study
Y. pseudotuberculosis IP32953 IP ΔYPTB0639	T6SS locus 4 gene YPTB0639 deleted and replaced with a Km resistance gene	This study
Y. pseudotuberculosis IP32953 IPmt ΔT6SS	IPmt T6SS locus 4 (YPTB0639-0657) deleted and replaced with a Km resistance gene	This study
Y. pseudotuberculosis IP32953 IPmt Δ*hcp*	Hcp gene of T6SS locus 4 (YPTB0463) deleted and replaced with a Km resistance gene	This study
Y. pseudotuberculosis IP32953 IPmt ΔYPTB0639	T6SS locus 4 gene YPTB0639 deleted and replaced with a Km resistance gene	This study
Y. pseudotuberculosis IP32953 IPmt Δ*ureD*	SY458 ΔYPTB1894-1891::*ymt* Δ*ureD* (*ym*t inserted into chromosome, urease-negative)	12, 14; this study
Y. pseudotuberculosis IP32953 IPmt Δ*psaA*	*psaA* deleted from IPmt Δ*ureD*	This study
E. coli S17-1 λ-*pir*	RP4 2-Tc::Mu-Km::Tn*7 pro thi recA* HsdR$^-$ M$^+$ λ*pir*	96
E. coli TOP10		Invitrogen
Plasmids		
pCH16	*Y. pestis ymt* gene (Y1069) cloned in pACYC177	13
pCR2.1-TOPO	High-copy-number cloning vector	Invitrogen
pCVD442	Suicide vector, Ap^r	86
pDS132	Suicide vector, Cm^r	97
pKD4	Mutagenesis; Ap^r, Km^r	88
pCP20	Flp recombinase gene, Ap^r, Cm^r	98
pCR::*lsrR-pstb*	*Y. pseudotuberculosis lsrR* gene (YPTB0553) cloned in pCR2.1	This study
pCR::YPTB3828	*Y. pseudotuberculosis* PDE4 gene (YPTB3828) cloned in pCR2.1	This study
pWKS130	Low-copy-number cloning vector	99

[a]-*pe*, *Y. pestis* allele; -*pstb*, *Y. pseudotuberculosis* allele; Ap, ampicillin; Km, kanamycin; Cm, chloramphenicol.

not *in vitro* (Fig. 3). Furthermore, replacement of the degraded and nonfunctional *ddhB-gmd* locus of *Y. pestis* with the functional *Y. pseudotuberculosis* homologs partially restored O-antigen production (see Fig. S1 in the supplemental material) and significantly reduced the normal autoaggregation phenotype of *Y. pestis* (Fig. 3). This *Y. pestis* strain (KIM6+ *ddhB-gmd*-pstb) also formed less biofilm *in vitro* and blocked a lower

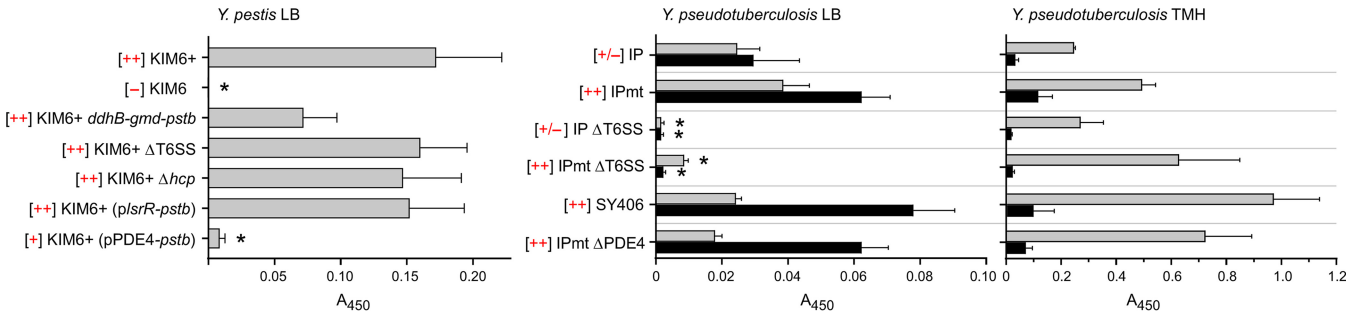

FIG 2 *In vitro* biofilm formation by *Y. pestis* and *Y. pseudotuberculosis* strains incubated at 21°C (gray bars) or 37°C (black bars) in LB or TMH media. Means and standard errors of results from three independent experiments performed in triplicate are indicated. *, $P < 0.05$ (relative to *Y. pestis* KIM6+ or *Y. pseudotuberculosis* IPmt by one-way ANOVA and Tukey's multiple-comparison test). The pigmentation phenotype of each strain on Congo red agar is given in brackets. "+" and "+/−" indicate levels of pigmentation intermediate between those full pigmentation (++; *Y. pestis* KIM6+) and nonpigmentation (−; *Y. pestis* KIM6).

percentage of infected fleas, although the differences were not statistically significant (Fig. 1 and 2).

Comparative transcriptomic analyses. To identify genes that may be important for regulating and forming biofilm in the flea, we compared the *in vivo* transcriptomic profiles of *Y. pestis* (CO92 strain, high flea blockage rate), a flea-transmissible *Y. pseudotuberculosis* mutant (IPmt strain, moderate flea blockage rate), and the *Y. pseudotuberculosis* wild-type parent strain (IP strain, unable to block fleas). The wild-type *Y. pseudotuberculosis* strain was first transformed with a plasmid containing the *Y. pestis ymt* gene (Table 1), enabling it to colonize the flea midgut to the same level as the other two strains (12). Microarray samples included total RNA isolated from bacteria recovered from the pooled digestive tracts of fleas dissected 1 day or 14 days after infection and from stationary-phase planktonic cultures incubated at 21°C, the same temperature at which the infected fleas were maintained. Only chromosomal genes were included in the analyses.

One day after infection, the fleas contained ~10^4 to 10^5 bacteria. At that time point, the bacteria have begun to multiply and form multicellular aggregates in the midgut and proventriculus, but mature biofilm has not yet developed (4). By 14 days, the blockage is at its peak incidence (5, 13, 21). Principal-component analyses (PCA)

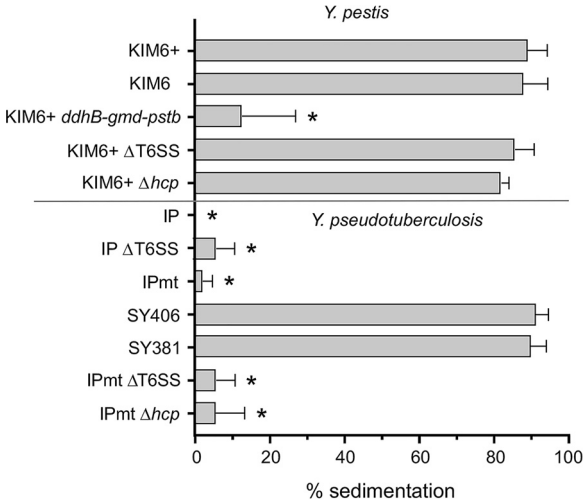

FIG 3 Autoaggregation of *Y. pestis* and *Y. pseudotuberculosis* strains during growth in LB at 21°C correlated with rough LPS production but not with Hms-dependent pigmentation or biofilm formation phenotypes. Strains able to produce O-antigen (*Y. pestis* KIM6+ *ddhD gmd pstb* and all *Y. pseudotuberculosis* strains except SY406 and SY381) showed significantly less sedimentation than *Y. pestis* KIM6+ (*, $P < 0.001$ [one-way ANOVA and Tukey's multiple-comparison test]).

indicated distinct expression patterns for *Y. pestis* compared to both *Y. pseudotuberculosis* strains under all conditions (Fig. S2). The two *Y. pseudotuberculosis* strains had very similar PCA patterns *in vitro*, but the patterns were more distinct in the flea. Pairwise comparisons of the *Y. pseudotuberculosis* wild-type strain and flea-blocking mutant and between each *Y. pseudotuberculosis* strain and *Y. pestis* were made using both flea and *in vitro* samples. Complete lists of all differentially expressed genes in the flea (≥2-fold difference in expression; $P \leq 0.05$ for intraspecific comparisons and $P \leq 0.01$for interspecific comparisons) are in Tables S1 to S6 in the supplemental material. Of particular interest were genes that were differentially regulated by the two *Yersinia* strains capable of biofilm-dependent blockage relative to wild-type *Y. pseudotuberculosis* (Table 2). A discussion of some of the more noteworthy differences follows.

Differential regulation of known biofilm-related genes. For both *Y. pestis* and *Y. pseudotuberculosis*, the bacterial biofilm that is required for stable colonization of the proventriculus and its eventual blockage depends on *hmsHFRS*-dependent synthesis and export of the exopolysaccharide that is a major component of the biofilm matrix (Fig. 1) (8, 9). Biofilm development in *Y. pestis* is temperature regulated both *in vitro* and in the flea, developing only at temperatures below about 26°C, which matches the flea environment. Wild-type *Y. pseudotuberculosis* never forms biofilm in the flea (12) (Fig. 1) but does so in different *in vitro* conditions (22). Temperature regulation of the HmsHFRS phenotype is posttranscriptional, and the operon is transcribed equivalently at low and high temperatures in *Y. pestis* (23–25).

Both in the flea and *in vitro*, *Y. pestis* expressed 6-fold to 19-fold-higher levels of the *hmsHFRS* genes than did either *Y. pseudotuberculosis* strain (Table 3). Even though the *Y. pseudotuberculosis* mutant is able to form a biofilm with proventricular blockage in the flea, it did not express higher levels of the *hmsHFRS* genes than the wild-type parent. This was despite the fact that the biofilm-inducing c-di-GMP diguanylate cyclase (DGC) genes *hmsT* and *hmsD* were more highly expressed in the flea 1 day after infection by both the *Y. pseudotuberculosis* mutant and *Y. pestis* than by *Y. pseudotuberculosis* (Tables 2 and 3). However, expression of *hmsD*, which has the predominant role in the flea (26), was greater in *Y. pestis* than in either *Y. pseudotuberculosis* strain on day 14 (Table 3). In contrast, three c-di-GMP-degrading phosphodiesterase (PDE) enzyme genes were equivalently expressed by *Y. pseudotuberculosis* and *Y. pestis* in the flea (Table 3). Two of them are pseudogenes in *Y. pestis*, and replacing the two functional homologs in *Y. pseudotuberculosis* with the nonfunctional *Y. pestis* alleles was essential for the ability of the *Y. pseudotuberculosis* IPmt strain to block fleas (12). A fourth PDE gene (YPTB3828; designated PDE4 here) is not efficiently translated due to the lack of a consensus ribosome binding site (27). However, transformation of *Y. pestis* with a high-copy-number plasmid harboring the *Y. pseudotuberculosis* gene resulted in a significant decrease in biofilm production *in vitro* (Fig. 2; *Y. pestis* KIM6+ [pPDE4-*pstb*] strain). Deletion of this gene in the *Y. pseudotuberculosis* mutant, however, did not significantly affect biofilm production (Fig. 2; IPmt ΔPDE4 strain). These differences in DGC and PDE c-di-GMP metabolic gene expression would be predicted to result in increased c-di-GMP levels in *Y. pestis* and contribute to higher levels of *hmsHFRS* expression.

The *Y. pestis* CO92 and *Y. pseudotuberculosis* IP32953 *hmsHFRS* operons share > 99.9% nucleotide sequence identity, but their modes of regulation evidently differ. The interspecies differences in *hms* gene expression or transcript stability that we observed could account for the lower flea-blocking potential of the IPmt *Y. pseudotuberculosis* strain than of *Y. pestis*. The reasons for these differences remain to be identified. Beyond the findings regarding c-di-GMP, little is known about the intermediate, more proximal regulation of *hmsHFRS* gene expression. In other bacteria, downstream effectors that bind c-di-GMP and regulate biofilm development include the PilZ domain and other protein families, mRNA riboswitches, and transcriptional regulators (28, 29).

TABLE 2 Genes differentially expressed by *Y. pestis* and the *Y. pseudotuberculosis* flea-transmissible mutant relative to the *Y. pseudotuberculosis* wild-type strain during infection of *X. cheopis* fleas[a]

Gene ID[b]		Gene name	Predicted gene function	Relative expression fold difference[c]	
				IPmt:IP	CO:IP
Day 1 after infection					
YPTB0570	YPO0425	*hmsT*	Diguanylate cyclase, c-di-GMP synthesis	2.3	2.4
YPTB0592	YPO0449	*hmsD*	Diguanylate cyclase, c-di-GMP synthesis	2.3	5.1
YPTB0728	**YPO3403**	***panD***	**Aspartate alpha-decarboxylase**	2.5	2.5
YPTB1108	**YPO2615**	***ybeJ***	**Glutamate and aspartate transporter subunit**	2.0	2.4
YPTB1923	**YPO1925**	***evg***	**Two-component response regulator**	2.6	2.6
YPTB3107	**YPO0863**		**YaeF/YiiX family of permuted papain-like enzymes**	2.2	2.4
YPTB3361	**YPO0700**		**Fimbrial protein**	3.2	3.0
YPTB3584	**YPO3646**	***pcp***	**Outer membrane lipoprotein SlyB**	3.4	2.6
YPTB0338	**YPO0281**	***hmuT***	**ABC transporter, periplasmic hemin-binding protein**	−4.9	−3.7
YPTB0339	**YPO0282**	***hmuS***	**Hemin degradation/transport protein**	−6.6	−5.0
YPTB0612	YPO0469	*dnaJ*	Chaperone protein	−2.6	−2.4
YPTB1515	**YPO1499**		**ABC transporter permease**	−3.4	−2.3
YPTB2113	**YPO2190**		**Ail-family outer membrane protein**	−4.0	−2.3
YPTB2239	YPO2320		XRE family transcriptional regulator	−2.4	−4.0
YPTB3475	YPO0582		YgjV family inner membrane protein	−3.0	−2.3
YPTB3905	YPO4084	*ibpB*	Heat shock chaperone	−2.9	−3.6
Day 14 after infection					
YPTB0728	**YPO3403**	***panD***	**Aspartate alpha-decarboxylase**	2.5	2.5
YPTB1107	YPO2614	*gltJ*	ABC glutamate/aspartate transporter, permease subunit	2.2	2.1
YPTB1108	**YPO2615**	***ybeJ***	**Glutamate and aspartate transporter subunit**	2.5	2.5
YPTB1522	YPO1507	*mglB*	Galactose binding periplasmic protein	2.1	2.9
YPTB1923	**YPO1925**	***evg***	**Two-component response regulator**	3.2	3.9
YPTB1934	YPO1936		Aminotransferase	2.0	7.8
YPTB1937	YPO1939		Glutaminase	4.8	3.3
YPTB1959	YPO1962	*argD*	Succinylornithine/acetylornithine transaminase	2.2	2.2
YPTB3107	**YPO0863**		**YaeF/YiiX family of permuted papain-like enzymes**	2.5	2.1
YPTB3361	**YPO0700**		**Fimbrial protein**	2.1	2.4
YPTB3534	YPO3699		PliI-like periplasmic lysozyme inhibitor	2.3	9.7
YPTB3582	YPO3648		2-Hydroxy-3-oxopropionate reductase	2.2	11.6
YPTB3584	**YPO3646**	***pcp***	**Outer membrane lipoprotein SlyB**	3.5	3.2
YPTB3736	YPO0165		LacI family transcriptional regulator	2.1	2.0
YPTB0110	YPO3927	*argC*	N-Acetyl-gamma-glutamyl-phosphate reductase	−5.0	−3.6
YPTB0111	YPO3925	*argB*	Acetylglutamate kinase	−3.0	−3.0
YPTB0336	YPO0279	*hmuV*	Hemin importer ATP-binding subunit	−3.4	−3.1
YPTB0337	YPO0280	*hmuU*	ABC hemin transporter, permease subunit	−4.1	−3.6
YPTB0338	**YPO0281**	***hmuT***	**ABC transporter, periplasmic hemin-binding protein**	−6.8	−6.1
YPTB0339	**YPO0282**	***hmuS***	**Hemin degradation/transport protein**	−9.5	−7.9
YPTB0526	YPO3446	*argI*	Ornithine carbamoyltransferase subunit I	−2.7	−2.8
YPTB1240	YPO1200	*adiC*	Arginine:agmatine antiporter	−2.5	−5.7
YPTB1241	YPO1201	*adiA*	Arginine decarboxylase	−2.0	−4.6
YPTB1423	YPO1398	*cspB*	Cold shock-like protein	−3.4	−3.0
YPTB1515	**YPO1499**		**ABC transporter permease**	−2.5	−3.7
YPTB1579	YPO1570	*argG*	Argininosuccinate synthase	−4.7	−2.9
YPTB2113	**YPO2190**		**Ail-family outer membrane protein**	−2.4	−4.4
YPTB2174	YPO2255	*araF*	Arabinose binding periplasmic protein	−2.0	−2.0
YPTB2954	YPO2652	*asr*	Acid shock protein precursor	−2.4	−8.3
YPTB3461	YPO0598		Putative hemolysin activator/exporter protein	−5.0	−5.0
YPTB3856	YPO4021		Biotin carboxylase superfamily protein	−2.8	−3.1

[a]Genes indicated in bold type were differentially regulated on both day 1 and day 14 after infection.
[b]Data represent annotation numbers of the *Y. pseudotuberculosis* IP32953 (YPTB) and *Y. pestis* CO92 (YPO) homologs.
[c]Data represent fold differences in expression by *Y. pseudotuberculosis* mutant (IPmt) and *Y. pestis* (CO) relative to the *Y. pseudotuberculosis* wild-type (IP) strain during infection of fleas.

Differential regulation of quorum sensing genes. The activation of quorum sensing (QS) systems coincides with the biofilm life stage in many bacteria, and QS systems control biofilm formation or disassembly in some cases (30–32). *Y. pseudotuberculosis* QS systems are involved in pathways leading to biofilm development on *Caenorhabditis elegans* (33). In the flea, genes in the *lsr* operon required for import of the autoinducer-2 QS signaling molecule LuxS were much more highly expressed by *Y.*

TABLE 3 Relative expression of known biofilm-related genes

gene	in the flea						in vitro		
	IPmt: IP		IPmt: CO92		IP: CO92		IPmt: IP	IPmt: CO92	IP: CO92
	d 1	d 14	d 1	d 14	d 1	d 14			
hmsH			-7	-8	-7	-13		-13	-19
hmsF			-6	-6	-6	-7		-10	-11
hmsR			-8	-9	-8	-12		-9	-11
hmsS			-12	-10	-10	-13		-9	-9
hmsT (DGC)	2				-2	-3	3		
hmsD (DGC)	2			-2	-5	-3		-2	-3
hmsC					-3	-3	2		-3
hmsP (PDE1)								2	
rtn (PDE2)*									2
YPTB3308 (PDE3)*								2	3
YPTB3828 (PDE4)				2		2		4	3
*rcsA**									
rcsB			2		2				
gmhA									
yfbA (YPTB2077)									

*pseudogene in *Y. pestis*

Key for Tables 3-8:

- between -2- to 2-fold difference (ns)
- 2-5-fold higher
- 2-5-fold lower
- 5-10-fold higher
- 5-10-fold lower
- >10-fold higher
- >10-fold lower

pestis than by either of the two *Y. pseudotuberculosis* strains (Table 4). This can be explained by the fact that the *lsrR* repressor is a pseudogene in *Y. pestis* but is intact in *Y. pseudotuberculosis* (34). The gene for the receptor for one of the autoinducer-1 QS signaling molecules, *ypsR*, was also significantly more highly expressed by *Y. pestis* (Table 4).

To determine if loss of *lsrR* contributed to the adaptation of *Y. pestis* to fleaborne transmission, we transformed *Y. pestis* with a high-copy-number plasmid harboring the functional *Y. pseudotuberculosis lsrR* gene. This restoration of LsrR function did not affect flea blockage or *in vitro* biofilm-forming ability, however (Fig. 1 and 2). These

TABLE 4 Relative expression of quorum sensing genes

gene	in the flea						in vitro		
	IPmt: IP		IPmt: CO92		IP: CO92		IPmt: IP	IPmt: CO92	IP: CO92
	d 1	d 14	d 1	d 14	d 1	d 14			
YPTB0547 *lsrG*			-6	-10	-8	-8		-7	-11
YPTB0548 *lsrF*			-6	-11	-7	-8		-8	-13
YPTB0549 *lsrB*			-6	-12	-10	-14		-7	-10
YPTB0550 *lsrD*			-5	-7	-5	-9		-6	-7
YPTB0551 *lsrC*			-15	-15	-28	-22		-11	-16
YPTB0552 *lsrA*			-19	-13	-18	-14		-10	-14
YPTB0554 *lsrK*			-6	-7	-7	-7		-3	-4
YPTB0830 *luxS*			2						
YPTB2499 *ypsI*								3	3
YPTB2500 *ypsR*			-3	-4	-3	-5		-4	-6
YPTB3258 *ytbI*									
YPTB3259 *ytbR*									

TABLE 5 Relative expression of fimbrial and outer surface protein genes

gene	in the flea						in vitro		
	IPmt: IP		IPmt: CO92		IP: CO92		IPmt: IP	IPmt: CO92	IP: CO92
	d 1	d 14	d 1	d 14	d 1	d 14			
psaE					2			3	3
psaF				4	9	4		4	4
psaA				81		103			2
psaB				5		7			
psaC								2	
ail			-4	-4	-4	-4			-2
ompX			4	3	3	2			
YPTB2113 (*ail2*)	-4	-2			2	4	-3		
YPTB3361	2	2			-3	-2	2		-2
YPTB3584 (*pcp*)	3	3			-3	-3	3		-2

results are consistent with our previous finding that deletion of the two autoinducer-1 QS systems and *luxS* did not affect proventricular blockage in the flea (6). Nevertheless, upregulation of QS systems could have been adaptive if they had enhanced dispersal of bacteria from the proventricular biofilm, thereby increasing regurgitative transmission efficiency.

Differential regulation of outer surface protein genes. Biofilm development normally begins with adherence to a surface and autoaggregation, a step typically mediated by fimbriae or other outer surface proteins. A major difference was seen in the expression levels of the *psa* genes that encode the pH 6 antigen, a fimbrial adhesin (Table 5). Psa expression in *Y. pestis* is repressed at low temperatures and induced at 37°C (35). Psa genes are even more highly repressed in infected fleas maintained at 21°C than in 21°C *in vitro* cultures, and the *Y. pestis* PhoPQ gene regulatory system is largely responsible for this repression (36, 37). Strikingly, the fimbrial structural gene *psaA* was expressed at levels 80-fold to 100-fold higher by the *Y. pseudotuberculosis* strains than by *Y. pestis* in fleas 14 days after infection (Table 5). The *psaEF* operon nucleotide sequences of the two species are identical and those of the *psaABC* loci >99.9% identical, but the *psaEF*-positive regulatory operon was 2-fold to 9-fold more highly expressed by *Y. pseudotuberculosis*. These results suggested that strong repression of the *psa* operon in the flea by *Y. pestis* was adaptive because producing these fimbriae is detrimental to the formation of a transmissible infection in the flea. To test this hypothesis, we examined the effect of *psaA* mutation on the flea-blocking ability of the *Y. pseudotuberculosis* mutant. Loss of the Psa fimbriae did not increase the blockage rate, however (Fig. 1; IPmt Δ*psaA* strain). A second fimbrial protein gene (YPTB3361/YPO0700) was more highly expressed in the flea 14 days after infection by both *Y. pestis* and the flea-blocking *Y. pseudotuberculosis* IPmt strain than by the *Y. pseudotuberculosis* IP parent (Tables 2 and 5).

Differential expression of members of the Ail/Lom family of outer surface protein genes was also detected. The *Y. pestis* Ail gene, an essential bubonic plague virulence factor (38, 39), was 4-fold more highly expressed in the flea than was *Y. pseudotuberculosis ail*. Ail is not required for the normal flea infection or blockage phenotype (38, 39); however, because it is required for resistance to the bactericidal complement activity of the mammalian innate immune system that it faces after flea bite transmission, upregulation of *ail* by *Y. pestis* in the flea may have been pathoadaptive. In contrast to the differential expression pattern of *ail*, the OmpX gene was more highly expressed by *Y. pseudotuberculosis* than by *Y. pestis*. OmpX has been shown to upregulate adhesin expression and biofilm formation in *Escherichia coli* under hyperosmotic conditions (40). Another member of the Ail family, YPTB2113/YPO2190, was downregulated during flea infection in *Y. pestis* and the flea-blocking *Y. pseudotuberculosis* IPmt strain compared to *Y. pseudotuberculosis* IP (Tables 2 and 5).

TABLE 6 Relative expression of transcriptional regulators[a]

gene	in the flea						in vitro		
	IPmt: IP		IPmt: CO92		IP: CO92		IPmt: IP	IPmt: CO92	IP: CO92
	d 1	d 14	d 1	d 14	d 1	d 14			
rovA		-2			2				
rovM		3		2			2	2	
YPTB1923 (*evg/bvg*)	3	3			-3	-4	3		-2
yitR			-6	-7	-18	-12		-67	-68
betI				8	19	13		2	4
YPTB3736		2				-2			

[a]Regulators that control expression of metabolic genes are listed in Table 7.

Differential expression of trancriptional regulators. Gene expression differences can stem from the presence, absence, or relative amounts of many different transcriptional regulators. In this category, the most disparate expression levels were seen for *yitR*, the transcriptional activator of the *yitABC* and *yipAB* insecticide-like toxin genes (41) (Table 6). It was observed previously that these genes are very highly upregulated and that the Yit/Yip proteins were correspondingly very highly produced by *Y. pestis* in the flea but not in liquid culture media incubated at the same temperature (36, 42, 43). Despite being members of the Toxin complex (Tc) family of insect toxins, the Yit/Yip proteins are not toxic to fleas; neither are they required for production of a normal infection in fleas (42, 43). Although the toxin genes differ between *Y. pestis* and *Y. pseudotuberculosis*, the *yitR* promoter regions are identical and the YitR protein is highly conserved between *Y. pestis* CO92 and *Y. pseudotuberculosis* IP32953 (286/288 amino acid identity). Thus, it is unclear why there should be such a large difference in expression of the YitR-activated genes in the two species. The *Y. pestis* Yit/Yip Tc genes inhibit phagocytosis by polymorphonuclear leukocytes and macrophages; thus, their upregulation in the flea may preadapt the bacteria to resist mammalian innate immunity immediately after transmission (36, 44).

During *Y. pestis* infection of the flea, the *rovM* transcriptional regulator gene is induced relative to the *rovA* virulence factor transcriptional activator gene; in contrast, *rovA* is induced during mammalian infection relative to *rovM* (25, 36). *rovM* expression was even higher in the flea-transmissible *Y. pseudotuberculosis* strain than in *Y. pestis* in the flea (Table 6). In both species, *rovM* is induced in nutrient-limited environments characteristic of the flea digestive tract (45, 46). Divergent results have been observed for the effect of RovM on biofilm formation during growth of the two species in nutrient-limited media, however; RovM reportedly activates biofilm development in *Y. pestis* but represses it in *Y. pseudotuberculosis* by negatively regulating *hmsHFRS* expression (46–48). Thus, differences in the RovM regulon may contribute to the lower flea blockage potential of the *Y. pseudotuberculosis* mutant strain.

The gene for the DNA-binding transcriptional regulator of a two-component gene regulatory system (YPO1925/YPTB1923) was upregulated by *Y. pestis* and the *Y. pseudotuberculosis* mutant in the flea compared to wild-type *Y. pseudotuberculosis* (Tables 2 and 6). This gene has similarity to *evgA*/*bvgA* of the EvgAS/BvgAS two-component system of *Escherichia coli*, *Shigella*, *Aeromonas*, *and Bordetella*. BvgAS has been shown to regulate biofilm development in *Bordetella* species (49, 50). Interestingly, the *evgS* downstream histidine kinase component gene is truncated in *Y. pestis*. Because the gene is upregulated in the two strains able to block fleas, it may be induced during biofilm development, and if it has a negative effect, loss or alteration of function of this two-component system in *Y. pestis* might have been selected. Another transcriptional regulator (YPTB3736/YPO0165) was upregulated by *Y. pestis* and the *Y. pseudotuberculosis* mutant 14 days after infection in the flea compared to wild-type *Y. pseudotuberculosis* (Tables 2 and 6).

Expression of *betI*, which encodes the repressor of the *betTBA* genes that govern synthesis of the osmoprotectant glycine betaine, was much higher in *Y. pseudotuberculosis* than in *Y. pestis*, suggesting that *Y. pestis* experiences greater osmotic stress in the flea. Consistent with this, the level of transcription of the *betT* and *proVWX* osmotic stress response genes was higher in *Y. pestis* than in *Y. pseudotuberculosis* (Table 7; see also Tables S4 and S6).

Differential expression of metabolism and stress response genes. Biofilm formation is typically induced in nutrient-limited environments and is part of an overall adaptive metabolic and physiologic response (51–53). The majority of the genes differentially regulated in the flea by *Y. pestis* versus *Y. pseudotuberculosis*, and by the two *Y. pseudotuberculosis* strains, encode metabolic proteins. Some of the major differences are shown in Tables 2 and 7.

The yersiniabactin iron acquisition system is an essential virulence factor of *Y. pestis*, but it is not required for infection of the flea gut (5). Nevertheless, the yersiniabactin genes were much more highly expressed by *Y. pestis* than by *Y. pseudotuberculosis* in the flea (Table 7), and such expression may promote *Y. pestis* infectivity in the mammal following transmission. A second iron acquisition system, *hmuSTUV*, was more highly expressed by the *Y. pseudotuberculosis* wild-type strain than by the flea-transmissible mutant strain or *Y. pestis* (Tables 2 and 7). The Hmu system takes up heme and heme-containing proteins rather than free iron (54), and the *hmuSTUV* operon, but not the *hmuR* outer membrane receptor gene, is expressed under iron-replete conditions by *Y. pseudotuberculosis* (55). Downregulation of *hmuSTUV* may indicate that hemin is not accessible to bacteria sequestered within a biofilm in the flea.

Certain metabolism genes previously noted to be highly upregulated by *Y. pestis* in the flea were not similarly upregulated by *Y. pseudotuberculosis*. These include a hydroxyphenylacetate uptake and utilization system involved in the metabolism of aromatic compounds (*hpa* genes) and the genes in chromosomal loci (YPO0622–0627 in *Y. pestis*; YPTB3427–3432 in *Y. pseudotuberculosis*) that include an *nhaC* Na^+/H^+ transporter, an aminotransferase, and two L-PSP family endonuclease genes predicted to inhibit translation (25, 36). Genes in this operon were 11-fold to 45-fold more highly expressed in the flea by *Y. pestis* (Table 7). However, deletion of this operon did not affect the ability of *Y. pestis* to block fleas (Fig. 1).

The glyoxylate pathway genes that enable use of acetate as a sole energy and carbon source were also comparatively highly expressed by *Y. pestis*, explainable by the fact that the *iclR* repressor of these genes is a pseudogene in *Y. pestis* but is functional in *Y. pseudotuberculosis*. Although the genes for the glyoxylate pathway are constitutively expressed, eliminating this metabolic capacity by deletion of the *aceA* isocitrate lyase gene was previously shown not to affect flea infection (56).

Y. pestis utilizes amino acids, particularly those corresponding to the L-glutamate family, as primary carbon and energy sources in the flea (36). Glutamate and aspartate transport genes were upregulated and arginine transport and biosynthesis genes downregulated by *Y. pestis* and *Y. pseudotuberculosis* IPmt in the flea relative to the *Y. pseudotuberculosis* IP parent strain (Tables 2 and 7). Histidine utilization pathway genes that act to convert histidine to glutamate, which are upregulated by *Y. pestis* in the flea (36), were even more highly upregulated by *Y. pseudotuberculosis*. This is of interest because histidine utilization correlates with increased biofilm development in *Y. pseudotuberculosis* and *Acinetobacter baumannii* (33, 57).

The urease genes were much more highly expressed in the flea by *Y. pseudotuberculosis* than by *Y. pestis*. *Y. pseudotuberculosis* urease activity is toxic to fleas, and mutational loss of the *ureD* gene in *Y. pestis* was evolutionarily important because it eliminated the high initial mortality of the flea vector that is counterproductive to the transmission cycle (14). A stronger acid stress response during infection of the flea was also observed in *Y. pseudotuberculosis* than in *Y. pestis*. This was particularly true for the *hdeB* gene, which encodes an acid-activated chaperone that protects proteins from low pH in the periplasm (58, 59).

TABLE 7 Relative expression of metabolism and stress response genes

gene	in the flea						in vitro		
	IPmt: IP		IPmt: CO92		IP: CO92		IPmt: IP	IPmt: CO92	IP: CO92
	d 1	d 14	d 1	d 14	d 1	d 14			
Iron acquisition systems									
hmuV		-3				3	-3		
hmuU	-3	-4				4	-4	-3	
hmuT	-5	-7			4	6	-5	-4	
hmuS	-7	-10			5	8	-6	-6	
hmuR				-2				-7	-5
ybt operon			-3 to -21	-3 to -12	-3 to -12	-3 to -29		-1 to -11	-1 to -11
ysuF			-2	-4	-2				-5
ysuC						2			
ysuJ									-10
Carbohydrate transport and metabolism									
araF		-2				2			
frwD		2		3					3
frwC		2		3					3
frwB				4				2	4
hpaR			-4	-4	-4	-5		-9	-11
hpaD			-3	-2		-2		-11	-11
hpaH			-3	-3		-4		-10	-10
hpaI				-3		-4		-7	-6
hpaX								-3	-3
hpaB								-2	-3
hpaC						-2			
mglB		2	-3			-3			
Amino acid transport and metabolism									
adiA		-2	3	2		5			
adiC		-2	3	2		6			
argB		-3	-2			3			
argC		-5	-3			4			
argG		-5	-3			3			
argI		-3	-8	-2		3			
gltJ		2				-2		-2	
hutG			2						
hutC								2	3
hutT			3		3				
hutH			6	3	6	2		2	5
hutU			9	4	10	3		3	4
panD	2	2			-2	-2	2		
proV				-3		-2		-2	
proW				-4		-2		-3	
proX				-3		-2		-2	
ureA			6		4				
ureB			3		2				
ureC			8	2	5	2			
ureE			8	3	5	3			
ureF			3	3	2	3		18	16
ureG			6	2		3			
ybeJ	2	2			-2	-2		-2	-2
Glyoxylate pathway									
aceK			-10	-10	-11	-11		-17	-12
aceA			-18	-5	-20	-18		-17	-12
aceB			-13	-8	-11	-12		-17	-13
Acid stress response									
hdeB			49	13	49	19		5	5
hdeD			2		2				
adiA		-2		2	4	5			
adiC		-2	3		5	6			
clcB			4		4	6		4	4
asr						3			
Miscellaneous									
YPTB1515	-3	-2			2	4	-2		
YPTB3107	2	2			-2	-2			
YPTB3427			-32	-16	-25	-26		-14	-17
YPTB3428			-17	-16	-15	-11		-14	-16
YPTB3429			-13	-12	-13	-12		-6	-7
YPTB3430			-37	-22	-39	-26		-33	-27
YPTB3431 *nhaC*			-45	-26	-31	-30		-50	-51
YPTB3432			-26	-19	-26	-30		-161	-170

TABLE 8 Relative expression of T6SS-4 genes

gene	in the flea						in vitro		
	IPmt: IP		IPmt: CO92		IP: CO92		IPmt: IP	IPmt: CO92	IP: CO92
	d 1	d 14	d 1	d 14	d 1	d 14			
YPTB0639	6	8	15	7			3		-3
YPTB0640 *tssC*		9		5			16		-21
YPTB0641 *tssB*	10	9	8	7			14		-19
YPTB0642 *impC*		7	8	7			11		-15
YPTB0643 *hcp*	13	6	24	4			17		-31
YPTB0644 *impF*		4		5			10		-16
YPTB0645 *tssF*		6		4			6		-11
YPTB0646 *tssG*		6		4			7		-8
YPTB0647 *clpB*		8		4			12		-9
YPTB0648 *vgrG*		5		5			7		-12
YPTB0649		7		4			7	-2	-24
YPTB0650		8		15			7		
YPTB0651		10		39			6		
YPTB0652		4		16			6		
YPTB0653 *tssJ*				11			7		
YPTB0654 *impL*		2		7			3		
YPTB0655 *impK*				7			16		
YPTB0656 *icmF*		2		5			14	3	
YPTB0657		4	11	13			11	11	

Differential expression of a type VI secretion system (T6SS). *Y. pseudotuberculosis* and *Y. pestis* have five chromosomal T6SS loci (60), and one of them, T6SS-4 (61), was highly upregulated in the flea by the *Y. pseudotuberculosis* IPmt strain compared to the wild-type parent IP strain or to *Y. pestis* (Table 8). *In vitro*, both the *Y. pseudotuberculosis* mutant and *Y. pestis* expressed higher levels of the T6SS-4 genes than did wild-type *Y. pseudotuberculosis*. Thus, consistent with previous findings, the expression of these genes is downregulated during flea infection compared to *in vitro* growth conditions by *Y. pestis* (36) but not by the flea-transmissible *Y. pseudotuberculosis* strain (Table 8). This T6SS was previously shown to be upregulated during *in vitro* growth at 26°C compared to 37°C in both species and to be upregulated by RovM and induced in response to diverse stresses in *Y. pseudotuberculosis* (61–68). In contrast, the *Y. pestis* T6SS-4 is upregulated by RovA (69). The T6SS-4 locus is highly conserved between the two species, although one of the 18 genes (YPTB0649) is split in *Y. pestis* CO92 (YPO0508/0509).

Previous work showed that the T6SS-4 is not required for *Y. pestis* to infect fleas (62). Here we show that the *Y. pestis* T6SS-4 is not required to produce biofilm in the flea or *in vitro* (Fig. 1 and 2). This was not the case for *Y. pseudotuberculosis*. Deletion of the T6SS-4 locus or of the single T6SS-4 *hcp* gene in the *Y. pseudotuberculosis* IPmt strain eliminated its ability to block fleas (Fig. 1). The T6SS-4 was also required by *Y. pseudotuberculosis* for normal biofilm formation in LB medium but not in TMH medium (Fig. 2). However, the T6SS-4 did not affect the Congo red agar pigmentation phenotype of *Y. pestis* or *Y. pseudotuberculosis* (Fig. 2). T6SS loci have been shown to directly or indirectly contribute to biofilm formation in other Gram-negative bacteria by as-yet-undefined mechanisms (70–73). The *Y. pseudotuberculosis* T6SS-4 has been reported to be upregulated by quorum sensing systems and in response to nutrient limitation and acidic, osmotic, and oxidative stress and to function in the transport of zinc ions (74), factors that may be relevant to biofilm growth. Of note, the Hcp protein of the *Y. pestis* T6SS-4 was reported to be an autoaggregation factor (75, 76). However, we found that deletion of this *hcp* gene or of the entire T6SS-4 locus of *Y. pestis* did not significantly affect its autoaggregative phenotype (Fig. 3).

Following growth in minimal media, Hcp or other T6SS proteins were not detected in culture supernatants (Fig. S3). Interestingly, however, PsaA appeared to be shed by the *Y. pseudotuberculosis* IP T6SS deletion mutants but not by the *Y. pseudotuberculosis* IPmt or *Y. pestis* T6SS deletion mutants. This suggests that under these *in vitro* conditions, the normal level of T6SS-4 expression is involved in Psa fimbrial stability or that mutations in the *rcsA* or c-di-GMP metabolic genes common to *Y. pestis* and *Y. pseudotuberculosis* IPmt indirectly affect PsaA synthesis or secretion posttranscriptionally. In nature, T6SSs act as interbacterial weapons that inject toxic proteins into adjacent bacteria (77). Thus, coregulation of biofilm formation and T6SSs may provide to *Y. pseudotuberculosis* a competitive advantage in environmental biofilms, which are usually mixtures with other bacteria. Genes of two other T6SSs were also uniquely upregulated by the biofilm-producing *Y. pseudotuberculosis* in the flea (see Table S3). In contrast, *Y. pestis* appears to have delinked biofilm and T6SS pathways.

Summary and conclusions. Microbial biofilm formation has often been considered a developmental process that involves initial attachment of planktonic cells to a surface followed by microcolony formation, structured macrocolony formation associated with an extracellular matrix, and dispersal (78). *Yersinia* biofilm in the flea differs in that the initial attachment is predominantly intercellular, resulting in bacterial autoaggregates associated with a heterogenous matrix derived from the flea blood meal, later to incorporate the bacterially derived polysaccharide matrix of the mature biofilm (4). The developmental model posits an ordered series of stage-specific genetic pathways during biofilm formation. However, comparative transcriptomic analyses of many different bacteria have not identified a comprehensive, stereotypical genetic program, even among closely related species (78–81). A build-up of intracellular c-di-GMP is a universal stimulus for biofilm development, but the forms of regulation of c-di-GMP flux and the intermediate steps leading to biofilm formation in different bacteria are many and varied (28, 29). The inducing signals and mechanisms underlying biofilm formation also differ among different bacteria, allowing each species to efficiently colonize its regular environmental niche (53, 82). *Y. pestis* and *Y. pseudotuberculosis* appear to exhibit species-specific pathways to infect and produce proventricular biofilm in the flea. Their expression profiles were distinct in the flea, reflecting different overall adaptive metabolic and physiological responses to the selfsame environment. These differentially expressed genes are not necessarily involved in biofilm formation *per se* but instead reflect adaptation to conditions in the flea gut and/or the biofilm state. For example, *rovM* is upregulated by both *Yersinia* species in response to nutrient limitations in the flea gut (45, 46) but differences in their RovM regulons may account for the dissimilar metabolic gene expression patterns and the reported opposite effects of RovM on biofilm development in the two species (46–48). During its evolutionary adaptation to fleaborne transmission, *Y. pestis* appears to have fine-tuned and remodeled the preexisting environmental biofilm induction pathways of its progenitor to match the specific environmental conditions of the flea gut.

MATERIALS AND METHODS

Bacterial strains and growth conditions for *in vitro* and *in vivo* transcriptome analyses. Wild-type *Y. pestis* CO92, wild-type *Y. pseudotuberculosis* IP32953 transformed with pCH16, a plasmid that harbors the *Y. pestis ymt* gene (referred to here as the IP strain) (83), and a modified *Y. pseudotuberculosis* IP32953 strain (IPmt) that is able to produce a transmissible infection in fleas (12) were used for gene expression analyses. The IPmt strain is identical to the IP strain except that the native *rcsA* (YPTB2486), phosphodiesterase-2 (PDE-2; *rtn*; YPTB1308), and phosphodiesterase-3 (PDE3; YPTB3308) genes were replaced by their nonfunctional *Y. pestis* pseudogene orthologues (Table 1) (12).

For *in vitro* samples, bacteria were grown from frozen stock in LB medium supplemented with 100 mM MOPS (morpholinepropanesulfonic acid) (pH 7.4) at 21°C without aeration to late log phase and subcultured (1:500) thrice in fresh LB/MOPS. A volume of the final cultures containing 5×10^8 to 1×10^9 stationary-phase bacteria (resulting from periods of approximately 24 to 30 h and 72 h of incubation for *Y. pseudotuberculosis* and *Y. pestis*, respectively) was centrifuged, all but 100 to 150 μl of the culture supernatant was removed, and the bacteria were resuspended in this and added to 10 volumes of RNAprotect Bacteria Reagent (Qiagen). After 10 min at room temperature, the samples were centrifuged for 5 min at 5,000 $\times$ *g*, the supernatant was removed, and the pellets were stored at −80°C until RNA isolation.

The *in vivo* bacterial samples were collected from digestive tracts of infected *Xenopsylla cheopis* fleas (36). Fleas were dissected 1 day or 14 to 15 days after infection in a drop of RNAprotect on a glass microscope slide and the digestive tracts removed. Digestive tracts from 25 to 400 fleas were pooled in a microcentrifuge tube containing 500 μl of RNAprotect, triturated manually with a small pestle to release the bacteria, and incubated for 10 min prior to centrifugation. Digestive tracts from 30 to 50 uninfected fleas were also collected. After centrifugation, the RNAprotect supernatant was removed and the pellets were stored at −80°C until RNA isolation.

RNA isolation, amplification, and microarray. Total RNA was isolated using an RNeasy Plus minikit (Qiagen). RNA was isolated from six independent *in vitro* samples and from four or six independent *in vivo* samples collected 1 day or 2 weeks after infection, respectively. As a control, RNA was isolated from three independent samples of uninfected flea digestive tracts. The absence of genomic DNA contamination was verified by PCR using primers matching the chromosomal *rtn* locus sequences that were identical in the two *Yersinia* species. RNA quality and integrity were verified using a model 2100 Bioanalyzer and the RNA 6000 Nano Kit assay (Agilent) and quantitated using a Qubit fluorometer and a Qubit RNA HS assay kit (Invitrogen). Purified RNA was stored at −80°C until use.

RNA amplification was carried out as previously described (36). Briefly, 100 ng of total RNA was amplified and labeled with modified biotin-16-UTP (Roche Molecular Biomedicals) and biotin-11-CTP (PerkinElmer) by using a MessageAmp II-Bacteria kit (Thermo Fisher Scientific). Amplified RNA was then fragmented using Ambion fragmentation reagents (Thermo Fisher Scientific) and hybridized to an RML custom Affymetrix GeneChip containing sequences for all *Y. pestis* CO92, *Y. pestis* KIM, and *Y. pseudotuberculosis* IP32953 predicted open reading frames (ORFs) and intergenic regions and scanned using an Affymetrix 7Gplus GeneChip scanner.

Microarray data analysis. Affymetrix GeneChip operating software (GCOS v1.4) was used for initial analysis of the microarray data at the probe set level. All **.cel* files, representing individual biological replicates, were normalized using the scaling method within Expression Console (v1.1.2800) to produce the summary-of-intensity **.chp* files. A pivot table with all samples was created that included calls, call *P* values, and signal intensities for each gene. The pivot table was then imported into GeneSpring GX 7.3, where hierarchical clustering (condition tree) using a Pearson correlation similarity measure with average linkage was used to produce the dendrogram indicating that biological replicates grouped together. The pivot table was also imported into Partek Genomics Suite software (Partek Inc., St. Louis, MO), log-transformed, and quantile-normalized to produce a principal-component analysis (PCA) plot as a second statistical test for the grouping of biological replicates. An analysis of variance (ANOVA) (2-way) test was run from this data set to produce *P* values for each comparison of interest, and the Benjamini-Hochberg method was used for multiple test correction.

The correlated replicates of all test conditions and controls were combined, and quality filters based on combined calls and signal intensities were used to further evaluate individual gene comparisons. Present and marginal calls were treated as the same, whereas absent calls were negatively weighted and eliminated from calculations. Ratios of test/control values and associated *t* test and ANOVA *P* values for all individual genes passing the filters described above were determined using GeneSpring and Partek software. Differential-expression parameters were set at a *P* value significance level of <0.05 and a fold change value of ≥ 2. Only chromosomal genes were included in this analysis. Interspecific gene comparisons were aided by use of the PSAT program (84). The microarray data determined in this work have been deposited in the NCBI GEO public database (85) and are accessible through GEO Series accession number GSE119243.

Construction of *Yersinia* mutants and complementation. In-frame deletion mutations in *Y. pestis* KIM6+, which lacks the *Yersinia* virulence plasmid but which blocks fleas as well as the fully virulent CO92 strain, and *Y. pseudotuberculosis* IP strains were constructed by allelic exchange using the pCVD442 suicide plasmid (86) containing a kanamycin (Km) resistance (Km^r) cassette flanked with long homology sequences of the target DNA (87) as previously described (14). Conjugative knockout plasmids (pCVDΔtarget::*km*) were introduced into *Escherichia coli* S17-1 λ-*pir* and transferred to *Y. pestis* and *Y. pseudotuberculosis* via conjugation. Positive transconjugants in which the deletion of the target genes had occurred were checked by PCR. The correct insertion of the Km cassette was verified with primer pairs encompassing one extremity of the Km cassette and the DNA region adjacent to the target gene. When necessary, the resulting Km^r mutants were transformed with the pCP20 plasmid encoding the Flp recombinase to remove the Km cassette (14). The Km cassette excision was verified by streaking isolated colonies simultaneously on LB agar plates with or without Km and by PCR. The pseudogenized *ddhD-gmd* region of *Y. pestis* KIM6+ was replaced with the fully functional *Y. pseudotuberculosis* homologs in five steps. The region was first deleted in *Y. pestis* and then reconstructed by incremental recombinative insertions of the *Y. pseudotuberculosis* homologs that had been amplified by PCR and cloned into the SphI and SacI sites of pCVD442. The O-antigen form (smooth versus rough) was characterized by Western blotting of total LPS extracts (see Fig. S1 in the supplemental material).

Deletion of *nhaC* locus genes y3550 to y3555 in *Y. pestis* KIM6+ was accomplished using a lambda Red recombinase system (88). PCR primers were designed to amplify a kanamycin resistance gene and to incorporate approximately 50 bp of genomic DNA homologous to regions on either side of the region to be deleted. After recombination, all six genes were deleted, including approximately 200 bp upstream of y3550 and 100 bp downstream of y3555.

YPTB0553 (*lsrR*) and YPTB3828 (PDE4) genes were amplified by PCR and cloned into pCR2.1-TOPO (Invitrogen). Deletion of YTPB3828 was accomplished using inverse PCR primers to delete amino acids 11 to 273 of 282 amino acids of the predicted open reading frame. After inverse PCR and religation, the

deletion fragment was moved from pCR2.1 to the pDS132 suicide vector using SphI and SalI sites and was moved into *Y. pestis* KIM6+ by allelic exchange after conjugation with *E. coli* S17-1.

Flea infection assays. Approximately 300 *X. cheopis* fleas were infected by allowing them to feed on blood containing ~5×10^8/ml *Y. pestis* CO92 or *Y. pestis* KIM6+ or containing *Y. pseudotuberculosis* IP32953 bacteria using an artificial feeding system as described previously (5). The infectious blood meal was prepared by growing the bacteria at 37°C in brain heart infusion (BHI) medium without aeration for ~16 h. A cell pellet containing ~10^9 bacteria was resuspended in 1 ml phosphate-buffered saline (PBS) and added to heparinized mouse blood or defibrinated rat blood (Bioreclamation). Fleas were allowed to feed for 1 h. Uninfected control fleas were fed on sterile blood. Fleas that took a blood meal were kept at 75% relative humidity and 21°C. Fleas infected with *Y. pseudotuberculosis* can experience acute toxicity due to urease activity (14), and only fleas that were healthy 24 h after infection were used. Fleas kept longer than 1 day were provided sterile maintenance blood meals twice weekly (89).

Flea proventricular blockage rates were assessed over a 4-week period after infection as previously described (5, 6). Flea infection rates and the average bacterial load per flea at 1 h or 28 days after infection were determined by CFU plate count results from samples of 15 to 20 fleas that were individually triturated and plated (5, 89). Blockage data were analyzed by using the two-tailed Fisher's exact test and infection data by Student's *t* test.

***In vitro* biofilm, pigmentation, and autoaggregation assays.** Bacteria were grown overnight in BHI broth and then diluted 1:100 into either LB media supplemented with 4 mM $CaCl_2$ and 4 mM $MgCl_2$ or TMH minimal media (90) and grown for 24 h at 21°C or 37°C. Cultures were then diluted 1:100 into the same media and grown again for 24 h at the same temperature. The cultures were diluted to an A_{600} of 0.02 in the same medium, and 100-μl aliquots were added to triplicate wells of 96-well polystyrene plates, which were incubated with shaking at 250 rpm for 24 h at temperature. The liquid, or planktonic phase, from each well was transferred to a new 96-well plate and measured at A_{600}. The wells of the 96-well plate with attached bacterial growth were washed four times with water, and the adherent biofilm was stained with 200 μl of 0.05% safranin for 15 min. The wells were washed four times with water, bound dye was solubilized with 200 μl of 30% acetic acid, and the A_{450} level was measured. Background absorbance values for uninoculated control wells were subtracted from the absorbance values corresponding to planktonic and biofilm growth. The absorbance values from triplicate wells were averaged as one data point for an average of two or three independent experiments. Results from three independent experiments performed with three replicates per experiment were analyzed by one-way analysis of variance (ANOVA) with Dunnett's posttest to compare the wild type to the other strains.

HmsHFRS-dependent pigmentation was determined by plating on standard Congo red agar (91) or on LB agar plates containing Congo red. Colony pigmentation was scored after 48 h of incubation at room temperature.

Autoaggregation was assessed by sedimentation assay (26). Bacteria from frozen stock were inoculated into BHI broth and grown overnight at 28°C and then subcultured to LB broth and grown overnight at 28°C. The LB culture was used to inoculate 8 ml of LB in a 15-ml tube, which was incubated at 21°C overnight with shaking at 250 rpm. Cultures were subjected to vortex mixing, a 1-ml volume was removed, and the optical density at 600 nm (OD_{600}) was recorded ($t = 0$). The remainder of the culture was allowed to sit undisturbed at room temperature for 3 h, and then 1 ml was removed from the top of the tube and the OD_{600} recorded. The 3-h OD_{600} value was divided by the $t = 0$ OD_{600} value to determine the percentage of sedimentation.

Secretion assays. *Yersinia* strains and mutants were grown at 28°C for ~16 h in BHI medium with aeration. Bacteria were pelleted from 2 ml of culture and washed twice in *Yersinia* defined minimal medium (YDM; 1× M9 minimal salts, 0.4% glucose, 0.4% Casamino Acids, 10 mM $MgCl_2$, 5 mM K_2SO_4, 10 μg/ml thiamine) (92), and a 100-μl aliquot was transferred to 10 ml fresh YDM. After 16 h of incubation at 28°C for 16 h, the culture was centrifuged and the supernatant filtered through a 0.2-μm-pore-size membrane and concentrated by using a 15-ml Amicon ultrafilter unit (3-kDa or 10-kDa cutoff). The cell pellets were washed in 1× PBS and resuspended in 1 ml 5 × SDS-PAGE loading buffer with beta-mercaptoethanol, and the proteins were separated by electrophoresis in a 4% to 20% gradient polyacrylamide gel and stained with Coomassie dye. The prominent protein in the culture supernatant fraction was identified by mass spectrometry (Protein Chemistry Section, Research Technologies Branch, NIAID, Bethesda, MD).

Data availability. The microarray data determined in this work have been deposited in the NCBI GEO public database (85) and are accessible through GEO Series accession number GSE119243.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mSystems.00217-18.

FIG S1, PDF file, 0.2 MB.
FIG S2, PDF file, 0.2 MB.
FIG S3, PDF file, 0.2 MB.
TABLE S1, PDF file, 0.3 MB.
TABLE S2, PDF file, 0.3 MB.
TABLE S3, PDF file, 0.4 MB.
TABLE S4, PDF file, 0.4 MB.
TABLE S5, PDF file, 0.4 MB.

TABLE S6, PDF file, 0.4 MB.
TABLE S7, PDF file, 0.2 MB.

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