Experimental evidence for negative selection in the evolution of a *Yersinia pestis* pseudogene

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***Yersinia pestis*, the agent of bubonic plague, evolved from the enteric pathogen *Yersinia pseudotuberculosis* within the past 20,000 years. Because ancestor and descendant both exist, it is possible to infer steps in molecular evolution by direct experimental approaches. The *Y. pestis* life cycle includes establishment of a biofilm within its vector, the flea. Although *Y. pseudotuberculosis* makes biofilms in other environments, it fails to do so in the insect. We show that *rcsA*, a negative regulator of biofilms that is functional in *Y. pseudotuberculosis*, is a pseudogene in *Y. pestis*. Replacement of the pseudogene with the functional *Y. pseudotuberculosis rcsA* allele strongly represses biofilm formation and essentially abolishes flea biofilms. The conversion of *rcsA* to a pseudogene during *Y. pestis* evolution, therefore, was a case of negative selection rather than neutral genetic drift.**

biofilm | flea | bubonic plague | phosphorelay | *Caenorhabditis elegans*

Evolution leaves pseudogenes in its wake. Pseudogenes are DNA sequences that have been inactivated by one or more mutations, but retain enough structure to be recognizable as the remnants of functional genes. Pseudogenes can be the result of neutral genetic drift: If a gene becomes unnecessary, random mutations accumulate in the absence of selection (1). A second possibility is that a pseudogene arises by negative selection when a functional gene becomes deleterious in a new environment.

Distinguishing between drift and negative selection experimentally is impossible in most cases because ancestral organisms are extinct. However, laboratory analysis is feasible for bacteria whose ancestors remain extant. A study of the enteric pathogen *Shigella flexneri* found that it lacks a large locus present in its ancestor, *Escherichia coli*. Heterologous expression in *S. flexneri* of a single *E. coli* gene from the locus reduced the pathogen's virulence, suggesting that negative selection was involved in the *S. flexneri* deletion (2).

Similar analysis, examining individual genes rather than large deletions, is possible using the bubonic plague bacterium *Yersinia pestis* and its ancestor, *Yersinia pseudotuberculosis*. Molecular evidence indicates that *Y. pestis* evolved from *Y. pseudotuberculosis* within the past 20,000 years (3), and DNA sequences of most of their common genes are at least 97% identical (4). Recently evolved pathogens are predicted on theoretical grounds to contain many pseudogenes (1). This was confirmed for *Y. pestis* by a comparison to *Y. pseudotuberculosis* that identified 208 putative *Y. pestis* pseudogenes, or ≈5% of the genome (4).

Plague is spread by flea bites. After feeding on a septicemic mammal, the insects can become "blocked" by a chronic *Y. pestis* infection in the digestive tract that prevents food from reaching the midgut. This starves the flea and prompts it to bite mammals repeatedly in futile attempts to feed, inoculating new hosts in the process. Not all infected fleas become blocked, and unblocked fleas are also capable of spreading disease, but it is nevertheless believed that blockage is crucial for long-term enzootic persistence of *Y. pestis* (5–7). Blockage is due to a biofilm that binds tightly in the proventriculus, an organ in the flea digestive tract; this makes the infection less susceptible to clearance by peristalsis and defecation and thus enhances high-titer colonization for extended periods (8, 9). The *in vivo* biofilm, consisting of bacteria in a self-synthesized, exopolysaccharide-rich matrix, requires the *hmsHFRS* operon of polysaccharide biosynthetic genes (8, 9). *Y. pseudotuberculosis* contains a functional *hmsHFRS* operon (10, 11), but it is unable to make biofilms in fleas (11). This suggests that the different biofilm capabilities in fleas are due to functions other than exopolysaccharide (EPS) biosynthesis.

Bacterial phosphorelays are signal transduction pathways that are similar biochemically to two-component systems but more complex (12–14). In two-component systems, a sensor histidine kinase autophosphorylates, then transfers the phosphate to an aspartate in the receiver domain of a response regulator. In phosphorelays, there are multiple signaling steps between these amino acids, e.g., His-Asp-His-Asp (12). The Rcs phosphorelay is conserved in Enterobacteriaceae, including *E. coli* and *Y. pestis* (15). The current understanding of Rcs, based on work in *E. coli*, is summarized in Fig. 1. At the end of the relay is RcsB, a DNA-binding protein activated by phosphorylation. RcsB regulates transcription of some targets on its own, whereas other targets require RcsA, an accessory protein that has no known RcsB-independent activity.

In this report, we show that *Y. pestis rcsA* is a pseudogene. Substitution with the functional homologue from *Y. pseudotuberculosis* abolishes biofilms in fleas, evidence that the *Y. pestis* pseudogene arose by negative selection. We also show that *rcsD*, despite an apparent frameshift that gives it the appearance of a pseudogene, is functional.

Results

***rcsB* Negatively Regulates Biofilms.** *Y. pestis* biofilm production is robust at 26°C but almost absent at 37°C (18). When grown at 26°C on agar media containing Congo red (CR), colonies adsorb the dye and become red, whereas at 37°C they remain white (19); the red phenotype correlates well, although not perfectly, with biofilm formation (20). By screening transposon mutants for aberrant red colonies at 37°C, Kirillina *et al.* identified a negative regulator of biofilms, *hmsP* (18). To identify additional negative regulators, we screened for mutants that made darker-than-normal red colonies at 25°C. From 4,000 transposon insertions examined, we obtained seven mutations: one in *hmsP*, five that will not be described here, and one in *rcsB*. We constructed a deletion strain, Δ*rcsB*, and it had the identical dark-red CR phenotype as the transposon mutant. Plasmid complementation produced colonies that were pink rather than red, presumably because of slight overexpression. These findings indicate that

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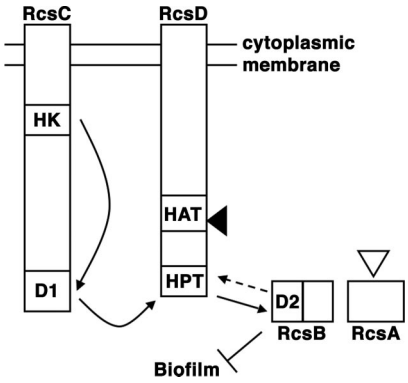

Fig. 1. Model of Rcs phosphorelay signaling. The histidine kinase domain (HK) of RcsC autophosphorylates, after which phosphate is transferred intramolecularly to aspartate in a receiver domain (D1). Next, phosphate is transferred intermolecularly from D1 to the histidine phosphotransfer domain (HPT) of RcsD. Last, phosphate is transferred from HPT to the RcsB receiver domain (D2). Biofilms are repressed by RcsB, and repression is increased by functional RcsA, as described in *Results*. Solid arrows show phosphate transfers that are well established experimentally (13, 14); dashed arrow indicates hypothesized phosphatase activity by RcsC, RcsD, or both (13, 16, 17). The histidine kinase-like ATPase (HAT) domain of RcsD does not participate in phosphotransfer. Filled arrowhead, site of frameshift in *Y. pestis* RcsD. Open arrowhead, site of inactivating insertion in *Y. pestis* RcsA.

rcsB is a negative regulator. *Y. pestis* RcsB is 91% identical to the well characterized *E. coli* protein and therefore is presumptively a transcriptional regulator.

Colonies of Δ*rcsB* were smaller than those of the wild type and had a rugose (wrinkled) morphology (Fig. 2). Studies in *Vibrio cholerae* indicate that the rugose phenotype requires EPS production (21); the Δ*rcsB* phenotype therefore suggests that EPS is overproduced. A strain defective for EPS, Δ*hmsS*, made smooth colonies slightly larger than those of the wild type (Fig. 2). *In vitro* biofilm assays confirmed that *rcsB* is a negative regulator: Biofilms made by Δ*rcsB* on polystyrene surfaces were substantially denser than those of the wild type (Fig. 3). Conversely, when *rcsB* expression was put under the control of an arabinose-inducible promoter, biofilms diminished with increasing concentration of inducer (Fig. 4).

Y. *pestis rcsA* Is a Pseudogene. *Y. pestis* YPO2449 has 25% amino acid identity to *E. coli* RcsA. Comparison with ancestral *Y. pseudotuberculosis* shows that YPO2449 contains a 30-bp internal duplication, resulting in a 10-aa insertion in an otherwise identical protein, and on this basis, YPO2449 was predicted to be a pseudogene (4). We refer to YPO2449 hereafter as *rcsA-pe* and to the *Y. pseudotuberculosis* allele as *rcsA-pstb.*

Deletion of a pseudogene should produce no phenotype, and when we deleted *rcsA-pe* from *Y. pestis*, there were indeed no changes in CR adsorption, colony size, colony morphology, and

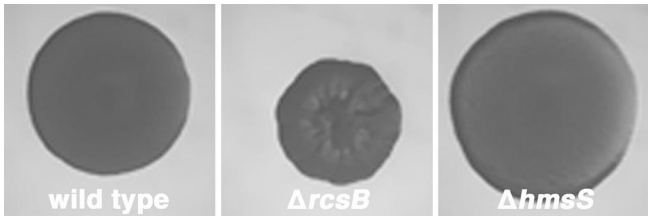

Fig. 2. Colony size and morphology of mutants. Δ*rcsB* overproduces biofilms and makes small, rugose (wrinkled) colonies. Δ*hmsS* is defective for biofilm formation and makes colonies slightly larger than those of the wild type. Typical colonies of each strain were photographed at identical magnifications.

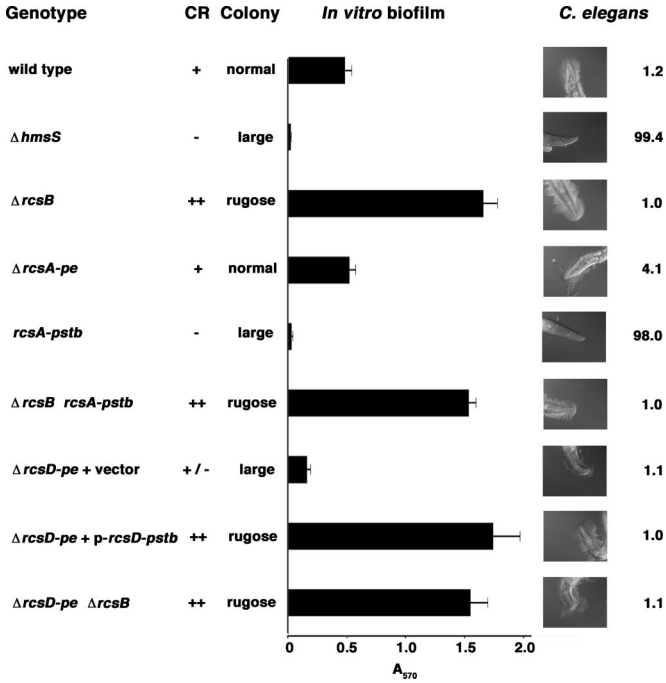

Fig. 3. Phenotypes of *rcs* mutants and substitutions. ''Δ'' indicates deletion; ''*-pe*'' and ''*-pstb*'' denote *Y. pestis* and *Y. pseudotuberculosis* alleles. Δ*rcsD-pe* experiments used the empty plasmid vector pET-32a(+); p-*rcsD-pstb* denotes the *Y. pseudotuberculosis* allele cloned into this plasmid under its native promoter. Congo red (CR) colony color phenotypes: −, white; +/−, pink; +, red; ++, dark red. Colony size and morphology are described in the Fig. 2 legend. *In vitro* biofilms grown on polystyrene culture dishes were quantified by staining with crystal violet (*Methods*); data are mean ± SD of two to four trials. Biofilms on *C. elegans* were photographed after overnight incubation on *Y. pestis* lawns; rare animals on *rcsA-pstb* acquired traces of biofilm (data not shown). Numbers are percentage of worms that grew to L4 stage in 2 d; data are aggregate of two to four trials in which a minimum of 1,100 worms were scored for each bacterial genotype.

in vitro biofilm growth (Fig. 3). Another assay for *Y. pestis* biofilms uses the nematode *Caenorhabditis elegans* (10, 22). Wild-type *Y. pestis* made biofilms on the worms' heads, which blocked feeding and inhibited growth, such that only 1.2% of eggs laid synchronously on bacterial lawns developed to the fourth larval stage (L4) in 2 days (Fig. 3). Slightly more nematodes (4.1%) developed normally on the Δ*rcsA-pe* strain. However, the growth inhibition by Δ*rcsA-pe* was still strong compared with that of the EPS-defective Δ*hmsS*, which made no detectable biofilms and permitted >99% of worms to reach L4.

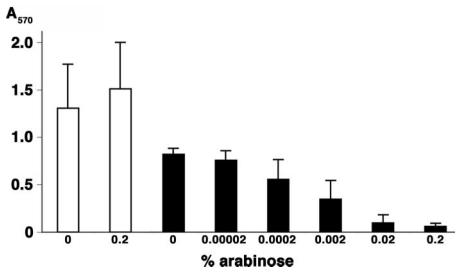

Fig. 4. *rcsB* represses biofilms. *In vitro* biofilms were grown in the Δ*rcsB* strain transformed with vector control (white) or *rcsB* (black) under an arabinose-inducible promoter. Inducer was added at indicated concentrations. Data are mean ± SD of two (vector) or three (*rcsB*) trials. The difference between control and *rcsB* samples in the absence of arabinose is apparently due to basal *rcsB* expression.

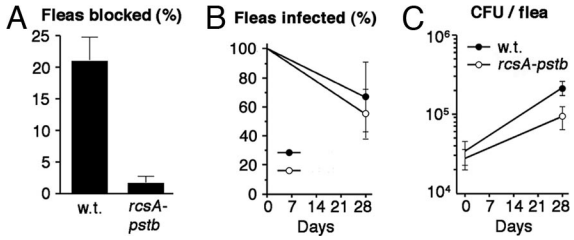

Fig. 5. Flea blockage and colonization by *rcsA-pstb*. Data are mean ± SEM of three trials. (*A*) Cumulative blockage frequency 4 weeks after infection with *Y. pestis*. At least 80 fleas were analyzed for each sample. (*B*) Percentage of fleas infected initially and after 4 weeks. (*C*) Colony-forming units (CFU) in fleas sampled immediately after infection (n = 20 per strain per trial) and in fleas that remained colonized after 4 weeks (n = 4–19) .

(Because wild-type *Y. pestis* inhibits growth almost completely, overproduction of biofilms by Δ*rcsB* or other mutants is not detectable with the *C. elegans* assay.)

In contrast to the deletion results, replacement of *rcsA-pe* with *rcsA-pstb* produced dramatic differences. *Y. pestis* with *rcsA-pstb* was negative for CR binding and made colonies resembling those of the EPS-defective mutant Δ*hmsS*, i.e., they were smooth and larger than wild-type colonies (Fig. 3). *In vitro* biofilms were absent, also like Δ*hmsS*. In the *C. elegans* assay, there was a slight inhibition of nematode growth: 98% of animals reached L4 with the *rcsA-pstb* substitution, whereas 99.4% did so on the EPS-defective Δ*hmsS* strain. Consistent with this weak growth inhibition, traces of extracellular matrix were observed on some worms exposed to *rcsA-pstb*, whereas none was ever present with Δ*hmsS*. The *rcsB* deletion was epistatic to *rcsA-pstb* for all phenotypes, indicating that in *Y. pestis*, as in *E. coli*, RcsA activity requires RcsB.

Functional RcsA Prevents Flea Blockage. To investigate the importance of the *rcsA-pe* pseudogene in its native environment, we experimentally infected the Oriental rat flea, *Xenopsylla cheopis.* With wild-type *Y. pestis*, 21% of fleas were blocked by biofilms (Fig. 5*A*). This is consistent with a previous study using the same *Y. pestis* background, in which 24–38% of fleas were blocked by the wild type, whereas none were blocked by an EPS-defective *hmsHFRS* mutant (8). The *rcsA-pstb* strain was severely defective for flea blockage (Fig. 4*A*), i.e., it resembles an EPS-defective strain (8, 9). The ability of the *rcsA-pstb* strain to establish a chronic infection was not compromised (Fig. 4*B*), and it grew in fleas to levels only slightly less than the wild type (Fig. 4*C*), indicating that the defect is specific for blockage.

To further establish the importance of *rcsA-pe* in *Y. pestis* evolution, we examined *rcsA* in *Y. pseudotuberculosis* biofilms. *Y. pseudotuberculosis* strain IP32953 (4) failed to produce detectable biofilms when adult *C. elegans* were incubated overnight on lawns of the bacteria. When *rcsA-pstb* was replaced with the nonfunctional *rcsA-pe* allele, robust biofilms were formed (Fig. 6). This indicates that RcsA negatively regulates biofilms in the ancestral organism and is additional evidence that conversion to a pseudogene was necessary in *Y. pestis* evolution.

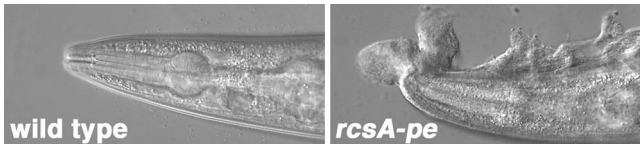

Fig. 6. RcsA negatively regulates *Y. pseudotuberculosis* biofilms. No biofilm formed on adult *C. elegans* placed on lawns of wild-type strain IP32953 and incubated overnight. Replacement of the wild-type allele *rcsA-pstb* with nonfunctional *rcsA-pe* resulted in the production of substantial biofilms.

***rcsD* Is Not a Pseudogene.** The *rcsD-pstb* ORF is 2,691 bp long, encoding a predicted 897-aa protein. The sequence includes a run of eight consecutive thymines (bases 1921–1928). The *Y. pestis* sequence is identical except that one thymine is deleted, causing a frameshift after codon 642. The frameshift is predicted to eliminate the HPT domain that directly participates in phosphorelay signaling (Fig. 1); it also disrupts the HAT domain, whose function is not known. Thus, the *Y. pestis rcsD-pe* allele has the appearance of a pseudogene.

We deleted most of *rcsD-pe* 5′ to the frameshift and found that biofilms were decreased, although less so than with Δ*hmsS* (Fig. 3). The existence of a mutant phenotype indicates that *rcsD-pe* is not a pseudogene, and the phenotype is consistent with a positive regulatory function. Substitution of *rcsD-pstb* for *rcsD-pe* slightly increased biofilms (data not shown), and plasmid-based expression of *rcsD-pstb* in the Δ*rcsD-pe* background greatly enhanced biofilms, also suggestive of positive regulation by RcsD (Fig. 3). A hypothesis that RcsD can have phosphatase activity on RcsB (13, 17) is consistent with this regulatory polarity. A Δ*rcsD-pe* Δ*rcsB* double mutant was fully derepressed, making biofilms equal to those of the Δ*rcsB* strain, indicating that RcsD regulation acts through RcsB.

Discussion

Because some bacteria coexist with their evolutionary ancestors, direct experimental comparisons are possible that can elucidate steps in molecular evolution. An early study found evidence for negative selection during *S. flexneri* evolution from *E. coli*. The *cadA* biosynthetic gene, part of a large *E. coli* locus that is missing in *S. flexneri*, was sufficient to reduce virulence when heterologously expressed in *S. flexneri* (2). Independent *cadA* losses in other *Shigella* sp. provided additional evidence for negative selection (23), and similar findings were made for other *Shigella* genes (24).

We used a similar approach to analyze a regulatory pathway in *Y. pestis* and its living ancestor, *Y. pseudotuberculosis.* We replaced the predicted *Y. pestis rcsA* pseudogene with the functional *Y. pseudotuberculosis* allele and also performed the reciprocal experiment, placing the pseudogene in *Y. pseudotuberculosis*. These experiments provided strong evidence that loss of functional *rcsA* was necessary for *Y. pestis* to occupy its unique niche in the flea vector.

Despite their recent divergence, the two *Yersinia* spp. differ markedly in disease severity, mode of transmission, and epidemiology. *Y. pseudotuberculosis* is not vector-borne, and it is not among the most virulent of pathogens. It is transmitted by the oral–fecal route and most often causes self-limiting gastroenteritis and lymphadenitis (25). *Y. pestis* is among the most lethal bacterial pathogens. It is spread by fleas, and this mode of transmission helps explain its extraordinary virulence: Only if a mammal is septicemic will a naïve flea that feeds on it become a vector (26).

The differences that allow *Y. pestis* but not *Y. pseudotuberculosis* to make biofilms in fleas are not completely known. Ymt, a *Y. pestis* factor encoded on a plasmid absent from *Y. pseudotuberculosis*, promotes survival in fleas, but it is not involved in biofilm formation *per se* (27). Ymt expression in *Y. pseudotuberculosis* was not sufficient to produce biofilms in the insect (11). *Y. pseudotuberculosis* produces robust *hmsHFRS*-dependent biofilms in the *C. elegans* model (10) and *in vitro* (11), indicating that the difference is not one of EPS production. *Y. pseudotuberculosis* is acutely toxic to fleas, whereas *Y. pestis* is not; although the genetic basis and mechanism of the toxicity are unknown, presumably it was lost in *Y. pestis* evolution (28).

The results presented here indicate that *Y. pestis* biofilm formation in fleas is due in part to an evolutionary change in the Rcs regulatory pathway. Heterologous expression of functional RcsA strongly represses *Y. pestis* biofilms in fleas and prevents blocking the insect digestive tract (Fig. 5). RcsA is functional in *Y. pseudo-*

tuberculosis and represses biofilms in the ancestral organism (Fig. 6). Conversion of *rcsA* to a pseudogene thus appears to have been required for *Y. pestis* to occupy the flea niche in a manner that promotes high transmissibility and enzootic persistence. We conclude that the pseudogene is a product of negative selection. Although several recent reports have shown that fleas can transmit plague without becoming blocked (5, 6, 29), the importance of blocked fleas in plague transmission is long established (7) and not refuted by those studies.

Y. pestis RcsB is functional and negatively regulates biofilms. Its deletion from the wild type results in small rugose colonies (Fig. 2) and aberrantly large biofilms *in vitro* (Fig. 3), whereas its overexpression produces large, smooth colonies (data not shown) and reduced biofilms *in vitro* (Fig. 4). RcsB is required for biofilm repression by functional RcsA (Fig. 3). The phenotypes of the *rcsA* and *rcsB* variants analyzed here are consistent with a model in which RcsB represses biofilms to some degree, but permits biofilm formation sufficiently to block fleas. When functional RcsA is present, RcsB-dependent repression is increased, such that essentially no biofilms are formed. This regulation appears to be indirect: We fused a *lacZ* reporter to chromosomal *hmsH* in the *rcsA-pe* and *rcsA-pstb* backgrounds and found no significant difference in expression of the EPS biosynthetic locus (data not shown).

rcsA-pe was predicted on bioinformatic grounds alone to be a pseudogene (4). Consistent with this hypothesis, there was no difference in colony phenotypes or *in vitro* biofilm formation in the Δ*rcsA-pe* strain. A slight defect was observed for *C. elegans* biofilms (Fig. 3), which could be interpreted to mean that wild-type *rcsA-pe* retains weak function. Alternatively, the result may indicate that *rcsA-pe* has a dominant-negative effect, i.e., the aberrant protein could be synthesized in the wild type and interfere with RcsB, lessening RcsB-mediated biofilm repression. This second interpretation is supported by the observation that the CR colony-color phenotype of the *rcsA-pstb* strain was partially rescued when *rcsA-pe* was overexpressed from a plasmid (data not shown).

Fully functional *rcsA-pstb* strongly repressed *Y. pestis* biofilms *in vitro* (Fig. 3), on *C. elegans* (Fig. 3) and in the natural niche, the flea (Fig. 5). *rcsA-pstb* bacteria colonized as many fleas as the wild type and grew in the insects to almost the same titers but failed to block the proventriculus. These phenotypes are virtually identical to those of an EPS-defective *hmsHFRS* mutant (8). We conclude that the failure to block fleas is specifically due to biofilm repression *in vivo*.

By inspection alone, *rcsD-pe* also appears to be a pseudogene: A single base pair is deleted, causing an apparent frame shift. However, *rcsD-pe* has some function, because biofilms are substantially reduced in the Δ*rcsD-pe* strain (Fig. 3). One possible explanation for RcsD function is suggested by the frameshift context, which occurs in a run of thymines; transcriptional slippage that changes reading frame is known to occur in polythymine tracts (30). Programmed translational frameshifting has also been observed in bacteria (31). Thus, it is possible that full-length RcsD is synthesized, perhaps at low levels, despite the putative frameshift. A second possibility is that the N-terminal portion of RcsD confers some function, perhaps as part of an interaction with RcsC. A hypothesis that RcsD has phosphatase activity on RcsB (13, 15, 17) is consistent with the regulatory polarity we observe; however, inhibition of RcsC signaling would also account for the *Y. pestis* effect. Regardless of the explanation, our findings indicate that caution is warranted in assigning pseudogene status on the basis of sequence data alone: *rcsA-pe* is a single ORF but is a pseudogene, whereas *rcsD-pe* is frameshifted yet functional.

The experimental approach described here is not limited to analysis of biofilms. The great differences between *Y. pestis* and *Y. pseudotuberculosis* in mammalian infection route and disease severity could also be due in part to conversion of genes to pseudogenes. Systematic substitution of *Y. pseudotuberculosis* functional genes for predicted *Y. pestis* pseudogenes, with tests in both flea and rodent models, could yield significant insights into the molecular evolution of an extraordinarily virulent pathogen.

Methods

***Y. pestis* Strains.** All strains used in this study are in the KIM6+ background, which is derived from the sequenced strain KIM (32) but cured of the pCD1/pYV plasmid required for mammalian virulence. It is competent for flea blockage (8) and *C. elegans* biofilms (10) and for convenience is referred to as wild type.

The Δ*hmsS* mutant and the substitution of *rcsA-pstb* for *rcsA-pe* were made by two-step allelic replacement (22, 33). *hmsS* was replaced with a chloramphenicol resistance gene. An unmarked substitution of *rcsA-pstb* for *rcsA-pe* was made by using a 2.2-kb PCR product amplified from *Y. pseudotuberculosis* YPIII that contained *rcsA-pstb* and flanking DNA; the sequence was verified to be identical to the *rcsA* gene YPTB2486 of the sequenced *Y. pseudotuberculosis* strain IP32953. The *rcsA-pe* substitution in *Y. pseudotuberculosis* was made in the same manner, by using a PCR product amplified from *Y. pestis*. Complete deletions of *rcsA-pe* (gene *y1741* in KIM; YPO2449 in *Y. pestis* strain CO92) and *rcsB* (*y2970*; YPO1218) and a deletion of nucleotides 1–1846 of *rcsD* (*y2968*; YPO1219) were made by using a one-step method to recombine PCR products into the chromosome (34). Strains made with this procedure were cured of the temperature-sensitive recombinase-encoding plasmid by overnight incubation at 37°C. Double mutants were made by sequential application of the procedures described. Oligonucleotide primers used are shown in supporting information (SI) Table S1. All strains were verified by PCR, Southern blot hybridization, DNA sequencing, and plasmid complementation, as appropriate. For inducible *rcsB* expression, the gene was cloned downstream of the arabinose-inducible promoter of plasmid pBADMycHis (Invitrogen). For plasmid expression of *rcsD-pstb*, the native promoter and ORF were cloned into vector pET-32a(+) (Novagen).

Screen for CR Phenotypes and Colony Morphology Assays. Transposon mutagenesis with Tn*5*-RL27 and identification of insertion sites was essentially as described (35). Mutants were plated on LBCR agar (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.01% Congo red, 1.5% agar) with 30 μg/ml of kanamycin and screened for color phenotypes after growth for 2 d at 25°C. To analyze colony size and morphology, strains were grown on the same medium, but with Congo red omitted, for 4 d at 25°C.

***In Vitro* Biofilm Assays.** Bacteria were grown in Luria–Bertani broth for 24 h and diluted to OD_{600} 0.3 in 40% brain-heart infusion broth. Four 750-μl aliquots per strain were added to wells of 24-well polystyrene dishes, which were incubated with shaking at 200 rpm for 16–18 h at 26°C. Media and planktonic cells were removed, the wells were washed with 2.5 ml of water, and the adherent biofilm was stained with 2.5 ml of 0.01% crystal violet for 12 min. The wells were washed three times with water, bound dye was solubilized with 1.5 ml of 80% ethanol-20% acetone, and the A_{570} was measured. Background absorbance for uninoculated control wells was subtracted.

***C. elegans* Biofilm Assays.** A previous method (22) was modified slightly to increase growth inhibition by *Y. pestis* biofilms. Gravid adult *C. elegans* were washed to remove *E. coli* on which they are fed, placed on lawns of *Y. pestis* for several hours to lay eggs, then removed. After incubation for 2 d at 20°C, growth of the broods to the fourth larval stage (L4) was scored. Micrographs of representative worms after overnight incubation on lawns were obtained by using differential interference contrast optics.

Nematode growth could not be used to assay *Y. pseudotuberculosis* IP32953 biofilms, because this strain has an uncharacterized activity that is lethal to unhatched *C. elegans* eggs. Therefore, *Y. pseudotuberculosis* biofilms were analyzed solely by placing adult *C. elegans* on bacterial lawns and incubating overnight.

Flea Blockage. Experiments were performed as described (8). *X. cheopis* fleas were fed a single blood meal containing *Y. pestis* and scored for blockage at twice-weekly intervals for 4 weeks. Additional samples of 20 fleas were collected immediately after the infectious blood meal, and at 4 weeks after infection, to determine the infection rate and bacterial load. Colony-forming unit (CFU) counts were made of individually triturated fleas.

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