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Evaluation of the Role of Constitutive Isocitrate Lyase Activity in *Yersinia pestis* Infection of the Flea Vector and Mammalian Host

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***Yersinia pestis*, unlike the closely related *Yersinia pseudotuberculosis*, constitutively produces isocitrate lyase (ICL). Here we show that the *Y. pestis aceA* homologue encodes ICL and is required for growth on acetate but not for flea infection or virulence in mice. Thus, deregulation of the glyoxylate pathway does not underlie the recent adaptation of *Y. pestis* to arthropod-borne transmission.**

The glyoxylate pathway is an anapleurotic cycle that bypasses the two CO_2-generating steps of the tricarboxylic acid (TCA) cycle to preserve carbon and so enables bacteria to use acetate or fatty acids as a sole energy and carbon source (4, 16). The glyoxylate cycle includes five TCA cycle enzymes and two specific enzymes: isocitrate lyase (ICL) and malate synthase (MDH) (16). In *Escherichia coli*, the glyoxylate bypass enzymes are encoded by the *aceBAK* operon which contains the genes for MDH (*aceB*), ICL (*aceA*), and the isocitrate dehydrogenase (ICD) kinase/phosphatase (*aceK*) (3, 15). The *aceBAK* operon is negatively regulated by the IclR repressor when carbon sources other than acetate and fatty acids are present and is derepressed by integration host factor when only acetate or fatty acid is available (32). Expression of the glyoxylate pathway is also controlled by the FruR and FadR transcriptional regulators and the small RNA-binding protein CsrA, and it is posttranscriptionally controlled by the phosphorylation of ICD (8, 17, 18, 21, 27, 35).

Yersinia pestis, the agent of plague, is unusual among *Enterobacteriaceae* because it constitutively produces the glyoxylate bypass enzymes (9, 10). Genome sequencing of *Y. pestis* revealed the presence of the potential *aceBAK* and *iclR* homologues, which are separated by 119 bp and are transcribed in the opposite direction (5, 25). The *Y. pestis aceA* gene is predicted to encode a protein of 345 amino acids with 54.4 to 84.4% identity and 68.3 to 89.4% similarity to ICL proteins of gram-positive and gram-negative bacteria (F. Sebbane, unpublished data). All the amino acids defined as essential for ICL activity in other bacteria (H184, H197, H356, K194, C195, S319, and S321) were conserved in *Y. pestis* (4). The *Y. pestis* gene *iclR* appeared to be a pseudogene due to a frame shift (5, 25). In contrast, the *iclR* homologue of the closely related *Yersinia pseudotuberculosis* (which, like other *Enterobacteriaceae*, has inducible ICL activity) is intact (1a, 10), suggesting that *iclR* mutation and constitutive expression of ICL provide a selective advantage important for the *Y. pestis* life cycle. Mutation of *iclR* appeared to be the only reason why *Y. pestis* constitutively produces ICL, because the upstream promoter region of the *Y. pestis aceBAK* homologue operon is identical to that of *Y. pseudotuberculosis* and contains predicted binding sites for IclR, FruR, and integration host factor.

Characterization of *Y. pestis aceA* mutants. To determine whether the *Y. pestis aceA* homologue encoded ICL, a *Y. pestis aceA* mutant was constructed by in-frame deletion. The *Y. pestis aceA* gene and upstream and downstream flanking regions were PCR amplified by using primer set A1 (5′GAACC TTGTTCTGGCGAG3′)–A2 (5′CACTGAGATCCCCTTTA GG3′) and cloned into pCRII to create pCJ1 (Table 1). Inverse PCR of pCJ1 was performed using primer set A3 (5′CTCGGCCTTATAGCGCCGAAGATGTCATCAAGCT GCGTG3′)–A4 (5′CCGCCAGCGTAATAAACTGGTATTT GTAGCCCATGGCGGAGA3′) to delete a 909-bp internal fragment of *aceA*, and the PCR product was ligated to generate pCJ2. A 1.5-kb XbaI-SacI fragment of pCJ2 containing the mutated *aceA* was subcloned into the suicide plasmid pCVD442 (6) to yield pCJ3, which was introduced by electroporation into *E. coli* S17-1λpir. The *aceA* deletion was introduced into the *Y. pestis* strains KIM6+ and 195/P by allelic exchange (6); the resulting mutants were named *Y. pestis* MacK and MacP, respectively. The 909-bp deletion in the two strains was verified by PCR analysis using primer sets that hybridized to sequences external (A1-A2) or internal (A5 [5′TTTGAAG ACCAATTGGCCGC3′]–A6 [5′CGGTCAGATTCTTCTTC CAG3′]) to the deleted region (Table 1, Fig. 1).

Loss of ICL activity in the *aceA* deletion strains was confirmed by enzymatic assay as previously described (26). No ICL activity was detected in the *aceA* mutants, in contrast to the wild type (Table 2). These results demonstrate that the *Y. pestis aceA* homologue encodes an ICL enzyme and imply that the *aceB* and *aceK* homologues encode the MDH and ICD enzymes.

It has been suggested that the constitutive glyoxylate pathway may compensate for TCA cycle or anapleurotic enzyme deficiencies in *Y. pestis* (1). To test these hypotheses, the ability to use glucose, pyruvate, and acetate as sole carbon and energy sources was investigated. *Y. pestis* KIM6+, but not the isogenic *aceA* mutant, was able to grow at 28°C on minimal media (25 mM HEPES [pH 7]; 2.5 mM K_2HPO_4, 10 mM NH_4Cl, 0.1 mM

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant property(ies)[a]	Reference or source
Y. pestis strains		
195/P	Wild type	2
KIM6+	pYV-negative strain, derived from KIM5	30
MacK	Derived from KIM6+ strain; Δ*aceA*	This work
MacP	Derived from 195/P strain; Δ*aceA*	This work
E. coli strains		
TOP10F′	F′ [*lacI*^{q} Tn*10* (Tet^r)] *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lac*X74 *recA1* *araD139* Δ(*ara-leu*)7697 *galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen
S17-1λpir	RP4-2-Tc::Mu-Km::Tn7/*pro hsd*R; host for pCVD442 and derivates	31
Plasmids		
pUC18	Cloning vector; Ap	34
pCRII	Cloning vector; Ap Km	Invitrogen
pCVD442	Suicide vector containing the counterselectable marker *sacB*; Ap	6
pCJ1	pCRIIΩ; ~2.4-kb PCR fragment generated with primer set A1-A2 encompassing the *Y. pestis aceA* gene and its upstream and downstream regions	This work
pCJ2	pCJ1 encompassing an internal deletion of 0.9 kb into *aceA* gene, which was created by PCR with primer set A3-A4	This work
pCJ3	pCVD442Ω; −1.5-kb XbaI/SacI insert from pCJ2	This work

[a] Ap, ampicillin resistance; Km, kanamycin resistance. Ω and Δ, insertion and deletion, respectively. pYV, *Yersinia* virulence plasmid.

$FeCl_2$, 0.01 mM $MnCl_2$, 20 mM $MgCl_2$, 2.5 mM $CaCl_2$, and 2.5 mM Na_2SO_3; 0.04 mM thiamine, 0.04 mM calcium pantothenate, and 0.04 mM biotine; 1 mM L-isoleucine, 1 mM L-valine, 1 mM L-phenylalanine, 1 mM L-methionine, and 5 mM L-glycine; and 1.5% agar) containing 0.2% acetate as a unique carbon source (Table 2). In contrast, both wild-type and mutant strains grew at comparable rates on minimal-medium plates containing 0.2% glucose or pyruvate. These results showed that ICL was required for use of the C_2 compound acetate but not the C_3 compound pyruvate, suggesting that *Y. pestis* has a fully functional TCA cycle; this conclusion is consistent with previous chemical and genetic analyses (7, 9, 25).

Effect of *aceA* mutation in a mouse infection model. ICL has been shown to be required by the intracellular pathogen *Mycobacterium tuberculosis* and the extracellular pathogen *Candida albicans* to survive in macrophages and establish infection in mammals (20, 22). Because *Y. pestis* is believed to be transiently phagocytized by macrophages or dendritic cells in the dermis after transmission (33), constitutive *aceA* expression could be important for pathogenesis. The impact of ICL loss was assessed by using a mouse experimental model of plague to test this hypothesis. The 50% lethal doses (LD_{50}s) (28) of *Y. pestis* 195/P and MacP were <10 CFU following intravenous inoculation and 46 and 32 CFU, respectively, following intra-

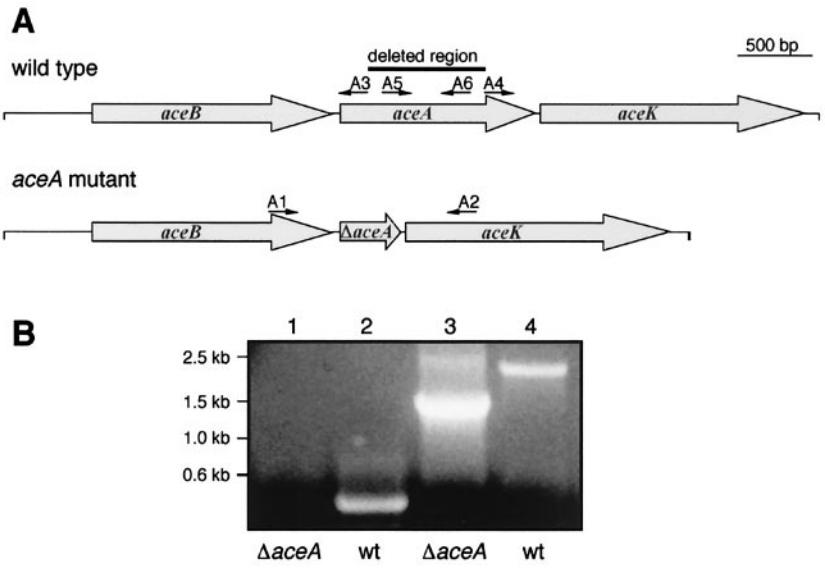

FIG. 1. Genetic organization of the chromosomal *aceBAK* operon and genetic analysis of the *aceA* mutant from *Y. pestis*. (A) Map of the *aceBAK* operon from wild-type *Y. pestis* and the isogenic mutants. (B) Agarose gel electrophoresis of amplicons obtained by PCR of wild-type and MacP DNA using primer sets A5-A6 (lanes 1 and 2) and A1-A2 (lanes 3 and 4). The results for the *Y. pestis* MacK mutant were identical.

TABLE 2. Comparison of wild-type and Δ*aceA Y. pestis*

Phenotype[a]	Wild type	*aceA* mutant
ICL activity	+	−
Growth on MM + glucose	+	+
Growth on MM + pyruvate	+	+
Growth on MM + acetate	+	−

[a] MM, minimal medium.

dermal inoculation. Moreover, no difference in time to disease onset was observed for animals inoculated with ICL-positive or -negative *Y. pestis* (Fig. 2).

Effect of *aceA* mutation in a flea experimental model. *Y. pestis* forms aggregates that resemble a biofilm in the flea midgut, which can involve the proventriculus and cause blockage, a crucial step for the bacterial transmission from fleas to mammals (13). In contrast, *Y. pseudotuberculosis* does not block the proventriculi of fleas (12). Because use of fatty acids, a major component of the flea's blood meal (19), requires the glyoxylate pathway, we tested whether the *Y. pestis* growth phenotype in the flea depends on ICL. *Xenopsylla cheopis* rat fleas were infected using a previously established flea model of infection (11). During the 4-week period after the infectious blood meal, 26% ± 5.6% of fleas infected with *Y. pestis* KIM6+ and 27% ± 2.1% of fleas infected with *Y. pestis* MacK developed proventricular blockage (Fig. 3A). No difference in the time to blockage was observed; fleas infected with either strain became blocked 2 to 4 weeks after infection. In addition, the infection rate and bacterial load achieved in the flea midgut were comparable (Fig. 3B). These results are in concordance with previous results showing that 22 to 38% of fleas infected with wild-type *Y. pestis* become blocked, that blockage appears 2 weeks after infection, and that infected fleas contain an average of 3×10^4, 1.8×10^5, and 5.2×10^5 bacteria at 0, 1, and 4 weeks after infection, respectively (12).

Conclusion. In mice *Y. pestis* virulence, unlike that of other pathogens, did not depend on ICL activity (20, 22), and ICL was not required to infect and block the flea vector either.

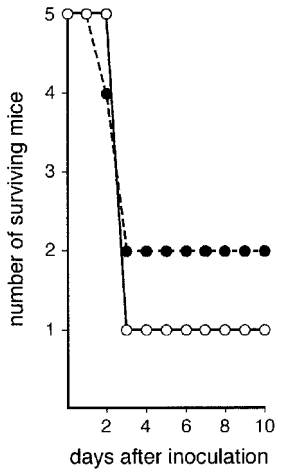

FIG. 2. Incidence of plague in mice injected intradermally with 96 CFU of *Y. pestis* 195/P (filled circles) or 150 CFU of the isogenic *aceA* mutant *Y. pestis* MacP (open circles).

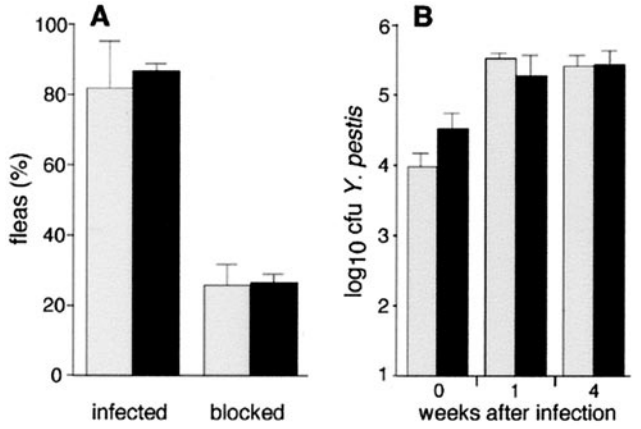

FIG. 3. Infection of *X. cheopis* fleas with *aceA*$^{+/-}$ *Y. pestis*. (A) Percentage of fleas infected and blocked. (B) Number of bacteria per infected flea during a 4-week period after a single infectious blood meal containing *Y. pestis* KIM6+ (gray bars) or the isogenic *aceA* mutant MacK (black bars). The averages and standard deviations of results from two independent experiments are shown, which included 198 fleas infected with KIM6+ and 194 fleas infected with MacK. Sample sizes of 8 to 20 infected fleas were used to determine the average bacterial loads shown in panel B.

Therefore, *Y. pestis* does not depend on fatty acids or acetate as an essential carbon source in the insect or mammalian host. In contrast to *Y. pseudotuberculosis*, none of more than 1,600 *Y. pestis* clones isolated from different animals and continents had a normally regulated glyoxylate cycle (10). Why does *Y. pestis* have a constitutive glyoxylate pathway, unlike its recent ancestor *Y. pseudotuberculosis*? Our results suggest that this deregulation is neither a burden nor an advantage. It has been proposed that *Y. pestis* is at an early stage of genome decay (36). Therefore, genes which are no longer required, like *aceBAK*, may remain as an inheritance from *Y. pseudotuberculosis*, and mutation of the *Y. pestis iclR* negative regulator may be an evolutionary accident with a neutral effect. However, it cannot be excluded that loss of *iclR* changes the regulation of genes other than *aceBAK* that are important for the *Y. pestis* flea-mammal cycle. Alternatively, constitutive ICL activity may be useful during saprophytic life, since *Y. pestis* has the capacity of long-term survival in soil (14, 23).

Two previous studies showed that genes which are intact in *Y. pseudotuberculosis* but are pseudogenes in *Y. pestis* (genes responsible for production of a smooth lipopolysaccharide and ureolytic activity) were not responsible for virulence differences between *Y. pestis* and *Y. pseudotuberculosis* (24, 29). In addition to these two examples of functional gene loss, our results show that loss of regulation of a biochemical pathway does not account for differences in pathogenesis between these two species.

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