Mutation of Phosphotransacetylase but Not Isocitrate Lyase Reduces the Virulence of Salmonella enterica Serovar Typhimurium in Mice

Yang Re Kim,† Shaun R. Brinsmade, Zheng Yang, Jorge Escalante-Semerena, and Joshua Fierer

VA Healthcare, San Diego, California; Department of Bacteriology, University of Wisconsin–Madison, Madison, Wisconsin; and Department of Medicine, University of California at San Diego School of Medicine, San Diego, California

Received 21 June 2005/Returned for modification 21 July 2005/Accepted 10 January 2006

A phosphotransacetylase (pta) mutant of Salmonella enterica serovar Typhimurium was attenuated in mice but survived normally in macrophages. Complementation of the pta mutation in trans restored virulence. An isocitrate lyase (aceA) mutant was virulent, so the inability to use acetate as a sole carbon source does not explain the phenotype.

Isocitrate lyase (Icl) catalyzes the first step in the glyoxylate shunt that enables many bacteria, including Salmonella enterica, to use acetate as both a carbon and an energy source (22). Munoz-Elias and McKinney recently reported that icl mutants of Mycobacterium tuberculosis are killed by mouse macrophages and are avirulent in mice (16). To determine whether isocitrate lyase (aceA) plays an analogous role in S. enterica pathogenesis, we made mutants of S. enterica serovar Typhimurium 14028s. The mutant cannot utilize acetate or propionate as a sole carbon source (Table 1). As controls, we inactivated the pta gene, which encodes phosphotransacetylase (Pta), the enzyme responsible for the interconversion of acetyl coenzyme A (CoA) and acetyl phosphate (1); the pta strain cannot grow in high acetate concentrations (~50 mM) (11, 21). The Pta enzyme also catalyzes the interconversion of propionyl-P and propionyl-CoA during the anaerobic degradation of L-threonine and during the catabolism of odd-chain fatty acids (7). Pta activity is needed to recapture propionate excreted by S. enterica during growth on 1,2-propanediol (17). In S. enterica, the expression of the 1,2-propanediol utilization (pdu) genes has been linked in vivo to expression studies to reduce fitness during infection (4). As a control for the inability to utilize propionate, we mutated prpB, which encodes the 2-methylisocitrate lyase that catalyzes the conversion of 2-methylisocitrate to pyruvate and succinic acid, a necessary step in the metabolism of propionic acid (6, 8). We were also prompted by the recently established connection between short-chain fatty acid catabolism and sirtuin (19) to investigate the role of cobB (sirtuin protein deacetylase) [20] in virulence.

There was no significant difference in the growth of aceA, prpB, and cobB mutants in LB broth at 37°C compared to strain 14028s, except that the cobB/pta and pta mutants had slightly increased lag times. All strains reached the same optical density by 8 h (Fig. 1). However, the growth of the cobB/pta and pta mutants was significantly reduced in LB broth under anaerobic conditions (10 ml of autoclaved broth injected into sterile Vacutainer tubes with methylene blue as an Eh indicator), and they reached only half the density of strain 14028s after 8 h. This did not occur in anaerobic Trypticase soy broth (TSB), which has added glucose, suggesting that pta is required for anaerobic growth on amino acids (7).

To test for virulence in mice, bacteria were grown in overnight at 37°C in TSB, washed twice in PBS, and resuspended in sterile saline. aceA and prpB mutants were fully virulent in genetically susceptible BALB/cJ that have a mutant Nramp1 (18) (Fig. 2A and B). In order to determine whether the aceA mutant was attenuated in immune mice, as was reported by McKinney et al. for M. tuberculosis isocitrate lyase 1 gene mutants (15), we infected congeneric, resistant BALB/c/Nramp1 mice that express the wild-type Nramp1 gene from DBA/2 mice (18). We extended the experiment to 21 days after infection, well into the acquired immunity phase of the infection. Neither the prpB nor the aceA mutants were attenuated in these resistant mice, as determined by enumerating the CFU in livers and spleens (Fig. 3). We concluded that, unlike M. tuberculosis (15), isocitrate lyase is not required for salmonelae to survive in immune mice and that the ability to utilize acetate or propionate as an energy source is not required for S. enterica virulence. This finding is in agreement with the recent report of Fang et al. that an aceA mutant of serovar Typhimurium was not attenuated when injected intraperitoneally into C3H/HeN mice (5). However, these authors found that prolonged survival of an aroA mutant of strain 14028s in the mesenteric lymph nodes of 129sv mice depended on aceA, although the effect was only apparent in these nodes and after 4 weeks (5).

Although the aceA mutant was virulent in both genetically resistant and susceptible mice, one of our control strains, the cobB/pta mutant, was attenuated in BALB/c mice (Fig. 2C). We then used P22 transduction to construct strains carrying each one of those mutations. The cobB/pta mutant was fully virulent (not shown), but the pta mutant was attenuated to the same extent as the double mutant (Fig. 2C). To establish the relationship between lethality and bacterial growth in vivo, we infected BALB/c mice with the pta mutant (95 CFU) and strain 14028s (65 CFU). Three days after infection there were 1,000-fold more viable 14028s than pta mutant organisms (5.25 ± 0.58 versus 2.18 ± 0.44), and all of the mice infected with strain

1 Corresponding author. Mailing address: VA Healthcare San Diego, 3350 La Jolla Village Dr., San Diego, CA 92161. Phone: (858) 552-7446. Fax: (858) 552-4398. E-mail: jfierer@ucsd.edu.
† Present address: Kangman St. Mary’s Hospital, Seoul, South Korea.
14028s were dead by day 4. Mice infected with the \textit{pta} mutant survived until day 6 after infection but by then they had $>10^6$ CFU/spleen, all of which were still resistant to antibiotics and unable to grow on acetate. These results show that although the \textit{pta} mutant grew more slowly in BALB/c mice than 14028s, it ultimately killed them. Thus, the \textit{pta} mutant was partially attenuated in these genetically susceptible mice.

We coinfected BALB/c.D2 mice intraperitoneally with equal numbers of the \textit{pta} mutant and strain 14028s, and after only 1 day there were more 14028s organisms in the spleens, although the difference in livers was not statistically significant. At all later time points there were only 2 to 6% as many \textit{pta} as 14028s in spleens and livers, and the differences were highly significant (Fig. 4). The \textit{pta} mutant was similarly impaired in BALB/c.D2 mice when the inoculum was grown to mid-log phase and if mice were infected intravenously (not shown). To confirm that the \textit{pta} mutation was responsible for the attenuation of the mutant, we complemented the mutation in \textit{trans} with the plasmid pPTA15 (Table 1), which restored the ability to grow on acetate and to grow in anaerobic LB broth. The complemented

14028s were dead by day 4. Mice infected with the \textit{pta} mutant survived until day 6 after infection but by then they had $>10^6$ CFU/spleen, all of which were still resistant to antibiotics and unable to grow on acetate. These results show that although the \textit{pta} mutant grew more slowly in BALB/c mice than 14028s, it ultimately killed them. Thus, the \textit{pta} mutant was partially attenuated in these genetically susceptible mice.

We coinfected BALB/c.D2 mice intraperitoneally with equal numbers of the \textit{pta} mutant and strain 14028s, and after only 1 day there were more 14028s organisms in the spleens, although the difference in livers was not statistically significant. At all later time points there were only 2 to 6% as many \textit{pta} as 14028s in spleens and livers, and the differences were highly significant (Fig. 4). The \textit{pta} mutant was similarly impaired in BALB/c.D2 mice when the inoculum was grown to mid-log phase and if mice were infected intravenously (not shown). To confirm that the \textit{pta} mutation was responsible for the attenuation of the mutant, we complemented the mutation in \textit{trans} with the plasmid pPTA15 (Table 1), which restored the ability to grow on acetate and to grow in anaerobic LB broth. The complemented

### Table 1. Strains used

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source(^c)</th>
<th>Lack of growth on various media</th>
</tr>
</thead>
<tbody>
<tr>
<td>General strains</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14028s</td>
<td>\textit{Salmonella enterica} serovar Typhimurium, wild strain</td>
<td>ATCC</td>
<td></td>
</tr>
<tr>
<td>JE4172</td>
<td>\textit{metE205 ara-9; accA112::MudJ}(^a)</td>
<td>JES</td>
<td>Acetate</td>
</tr>
<tr>
<td>JE3056</td>
<td>\textit{metE205 ara-9; prpB121::Tn10d(tet(^r))}(^b)</td>
<td>JES</td>
<td>Propionate</td>
</tr>
<tr>
<td>JE4718</td>
<td>\textit{metE205 ara-9; cobB1176::Tn10d(tet(^r)) pta102::MudJ}</td>
<td>JES</td>
<td>Acetate and propionate</td>
</tr>
<tr>
<td>14028s derivatives</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YR2001</td>
<td>\textit{aceA112::MudJ}</td>
<td>This study</td>
<td>Acetate</td>
</tr>
<tr>
<td>YR2002</td>
<td>\textit{prpB121::Tn10d(tet(^r))}</td>
<td>This study</td>
<td>Propionate</td>
</tr>
<tr>
<td>YR2003</td>
<td>\textit{cobB1176::Tn10d(tet(^r)) and pta102::MudJ}</td>
<td>This study</td>
<td>Acetate and propionate</td>
</tr>
<tr>
<td>YR2004</td>
<td>\textit{pta102::MudJ}</td>
<td>This study</td>
<td>Acetate</td>
</tr>
<tr>
<td>YR2005</td>
<td>\textit{cobB1176::Tn10d(tet(^r))}</td>
<td>This study</td>
<td>Acetate and acetate</td>
</tr>
<tr>
<td>YR2006</td>
<td>\textit{pta102::MudJ/pPTA15 Para(^{EX})-pta(^r)}</td>
<td>This study</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Abbreviation for MudI\(1734\) (2).
\(^b\) Abbreviation of Tn\(10d\)\(16\)\(H11004\)\(17\) (24).
\(^c\) ATCC, American Type Culture Collection; JES, J. Escalante-Semerena.

![Growth curves for strain 14028s and for five mutants grown aerobically and anaerobically in LB and TSB at 37°C. Anaerobic conditions were achieved by boiling the media to drive off dissolved air and injecting the hot broth into sterile, stoppered 16-by-125-mm vacuum tubes (Vacutainer; BD). The anaerobic growth of \textit{pta} mutant in LB broth was slower and still did not reach the level of the control strain even after 24 h.](image-url)
FIG. 3. Isocitrate lyase (aceA) and prpB are not required for virulence in genetically resistant BALB/c.D2 mice. Mice were infected intraperitoneally with $2 \times 10^8$ 14028s organisms or one of the mutants. Six mice in each group were sacrificed on days 5 (not shown), 14, and 21 after infection. There were no significant differences between CFU counts in either the spleens or the livers of mice infected with the two strains at any of these time points.

FIG. 2. Survival curves for BALB/c mice infected intraperitoneally with $\sim 30$ CFU of either strain 14028s or one of the mutants. The survival curves for the prpB (A) and aceA (B) mutants did not differ significantly from the control organism, strain 14028s (eight mice/group). (C) Mice ($n = 15$ to 17) infected with the cob/pta or pta mutant survived significantly longer than the mice infected with strain 14028s ($P < 0.01$). There was no significant difference in the survival of mice infected with the cob/pta double mutant and the pta mutant ($P > 0.2$).
strain was also virulent in BALB/c.D2 mice; the competitive index (CI) for the complemented strain was nearly 0 (Fig. 5).

Since *S. enterica* is a facultative intracellular pathogen that grows inside macrophages, we determined the effect of the *pta* mutation on survival inside periodate-elicited peritoneal macrophages. We opsonized bacteria with 20% normal human serum and coinfected adherent macrophages for 30 min with the 14028 and *pta* mutant strains. After 0, 4, and 18 h of incubation in Dulbecco modified Eagle medium with 20 μg of gentamicin/ml, we lysed the macrophages to determine the surviving CFU. We did not find a difference in survival between the 14028s strain and the *pta* mutant (data not shown). This confirms the recent report of Kim and Falkow (10) that a *pta* mutant is not more susceptible to macrophage killing.

Lawhon et al. did not find that *S. enterica pta* mutants are attenuated in mice (12), so we expected the *pta* mutant to be a negative control in these experiments. We cannot be sure why our results differ from theirs, but they used a double *ackA-pta* mutant and they tested virulence only in BALB/c mice by determining the oral 50% lethal dose (12). Since we found that the *pta* strain killed BALB/c mice, although at a slower rate, it is possible that Lawhon et al. overlooked the attenuation of the mutant in an oral infection model, in which there tends to be greater variation in time to death within a group. It is also possible that the *ackA* (acetate kinase) mutation affected virulence in some way. AckA is the enzyme that phosphorylates short-chain fatty acids (i.e., acetate and propionate), yielding acetyl-P, which is in turn converted to the CoA derivative by Pta. During growth on acetogenic substrates (e.g., glucose), *pta* mutants have no acetyl-P, whereas *ack* mutants accumulate acetyl-P (25). We can only speculate on why the *pta* mutation attenuates serovar Typhimurium infections in mice. Attenuation is not due only to their inability to use acetate for energy, since the *aceA* mutant was virulent. It is possible that Pta has some other function in *Salmonella* and that is currently under investigation. It has also been claimed that acetyl-P can act as a P donor and thus play a role in signal transduction (23, 25). Acetyl-P can phosphorylate OmpR, leading to the repression of flagellum synthesis in *Escherichia coli* (14) and *pta* mutants are hyperflagellated. However, McCleary determined that the kinetics of phosphorylation of PhoB by acetyl-P made it un-
likely that acetyl—P served that function in vivo (13). There are many two-component regulators in S. enterica, and we cannot exclude that acetyl—P plays a physiological role in activating one or more of them in S. enterica. Indeed, Chamnongpol and Groisman showed that acetyl—P can donate a phosphate to a mutant PhoP protein that functions in the absence of the sensor PhoQ and that activity requires Pta (3). They also found that the native PhoP protein could be phosphorylated by acetyl—P, although less efficiently. The exact mechanism of attenuation in the pta mutant remains to be determined.

We thank Bruce Zwilling for breeding pairs of the BALB/c.D2 Numo1 congenic mice and Sharon Okamoto for technical assistance. This work was supported by NIH grants R01 AI47884 (J.E.-S.) and R01 GM40313 (J.E.-S.).

REFERENCES


Editor: F. C. Fang