Isocitrate Lyase Activity Is Required for Virulence of the Intracellular Pathogen *Rhodococcus equi*

Daniel M. Wall, Pamela S. Duffy, † Chris DuPont, John F. Prescott, and Wim G. Meijer

Department of Industrial Microbiology, Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Dublin 4, Ireland, and Department of Pathobiology, University of Guelph, Guelph, Ontario N1G 2W1, Canada

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*Rhodococcus equi* is an important pathogen of foals, causing severe pyogranulomatous pneumonia. Virulent *R. equi* strains grow within macrophages, a process which remains poorly characterized. A potential source of carbon for intramacrophage *R. equi* is membrane lipid-derived fatty acids, which following β oxidation are assimilated via the glyoxylate bypass. To assess the importance of isocitrate lyase, the first enzyme of the glyoxylate bypass, in virulence of a foal isolate of *R. equi*, a mutant was constructed by a strategy of single homologous recombination using a suicide plasmid containing an internal fragment of the *R. equi* aceA gene encoding isocitrate lyase. Complementation of the resulting mutant with *aceA* showed that the mutant was specific for this gene. Assessment of virulence in a mouse macrophage cell line showed that the mutant was killed, in contrast to the parent strain. Studies in the liver of intravenously infected mice showed enhanced clearance of the mutant. When four 3-week-old foals were infected intrabronchially, the *aceA* mutant was completely attenuated, in contrast to the parent strain. In conclusion, the *aceA* gene was shown to be essential for virulence of *R. equi*, suggesting that membrane lipids may be an important source of carbon for phagocytosed *R. equi*.

The gram-positive bacterium *Rhodococcus equi* is a major cause of subacute or chronic granulomatous bronchopneumonia in young foals up to 5 months in age and sporadically infects other animals, such as pigs, goats, and cattle. In addition to its animal hosts, *R. equi* is increasingly responsible for AIDS-associated pneumonia in human immunodeficiency virus-infected individuals (26). The virulence of *R. equi* is dependent on its ability to infect and proliferate in macrophages, eventually resulting in necrotic death of the infected cell (13, 22). Intramacrophage *R. equi* appears to be located exclusively within membrane-enclosed vacuoles and persists coiled with the absence of phagosome-lysosome fusion (11, 41). All virulent strains isolated from horses contain an 80- to 85-kb plasmid, which is essential for replication and cytotoxicity of the pathogen in macrophages (8, 22, 35, 36). A detailed analysis of the nucleotide sequence of the virulence plasmid of two clinical isolates from foals revealed the presence of a 27-kb pathogenicity island, harboring at least 21 genes (34). One of these genes, encoding the lip-modified, surface-expressed, virulence-associated protein A (VapA), has been shown to be essential for virulence (18, 33, 37). The pathogenicity island contains an additional seven *vapA* homologues, two of which are pseudogenes (5, 30, 34). Expression of the VapA gene and other genes in the pathogenicity island is controlled by a range of environmental parameters, including temperature, pH, oxidative stress, and the concentrations of calcium and magnesium (1, 5, 29, 33).

A major area of interest is the determination of what sources of carbon sustain bacterial or fungal pathogens following infection. It has been shown that in contrast to cells grown in liquid medium, *Mycobacterium tuberculosis* isolated from lungs of infected mice actively oxidizes fatty acids, indicating that these may be an important source of carbon for this pathogen (3). Fatty-acid metabolism proceeds via the dissimulation of these substrates via β oxidation, followed by the assimilation of the resulting acetyl-coenzyme A (CoA) via the glyoxylate shunt. The latter pathway consists of the combined activities of isocitrate lyase and malate synthase, converting isocitrate and acetyl-CoA to succinate and malate. This pathway therefore circumvents the two decarboxylation steps of the citric acid cycle, allowing assimilation of two carbon (C₂) substrates, such as acetate, ethanol, and fatty acids (20). A major breakthrough in the analysis of the physiology of intracellular pathogens was the detection of isocitrate lyase mRNA and protein in phagocytosed *M. tuberculosis* and *Mycobacterium avium*, followed by the observation that an isocitrate lyase-deficient mutant of *M. tuberculosis* is impaired in persistence in macrophages and mice (10, 14, 25). Interestingly, the requirement for isocitrate lyase was apparent largely in immunocompetent mice, and the mutant was markedly attenuated for survival in activated but not in resting macrophages. These observations lead to the suggestion that lipids, derived from either macrophage membranes or cell remnants in granulomas, are a major source of carbon for *M. tuberculosis* (25). Since then, the glyoxylate bypass has been shown to be important for virulence of *Rhodococcus fascians* and the fungal pathogens *Candida albicans*, *Leptosphaeria maculans*, and *Magnaporthe grisea* (17, 21, 38, 39). Interestingly, isocitrate lyase-deficient mutants of *Cryptococcus neoformans* and *Saccharomyces cerevisiae* are not affected in virulence (9). We recently characterized the gene encoding isocitrate lyase of *R. equi* (19). The aim of the current...
paper was to determine the role of isocitrate lyase in virulence of \( R. \ equi \) in foals.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** Plasmids and strains used in this study are listed in Table 1.

**Media and growth conditions.** Bacterial strains were grown in Luria-Bertani (LB) broth (31) or in \( R. \ equi \) minimal medium containing either succinate (20 mM), acetate (20 mM), or lactate (20 mM) as a source of carbon (19). Where appropriate, the following supplements were added: kanamycin, 50 \( \mu \)g mL\(^{-1}\) (\( E.\ coli \)) or 200 \( \mu \)g mL\(^{-1}\) (\( R. \ equi \)); ampicillin, 80 \( \mu \)g mL\(^{-1}\); 5-bromo-4-chloro-3-indolyl-\( \beta \)-D-galactopyranoside, 20 \( \mu \)g mL\(^{-1}\); isopropyl-\( \beta \)-D-thiogalactopyranoside, 0.1 mM. For solid media, agar was added to 1.5% (wt/vol).

**DNA manipulations.** Plasmid DNA was isolated with the alkaline lysis method (2). DNA-modifying enzymes were used according to the manufacturer’s recommendations (Roche). Dideoxy sequencing reactions were done with the CEQ DCTS kit as described by the manufacturer (Beckman). The nucleotide sequence was determined using a Beckman CEQ 8000 automatic sequencer; nucleotide sequence data were compiled using the Staden package (32). PCR was carried out using Taq DNA polymerase (Promega) or Deep Vent DNA polymerase (New England Biolabs) as described by the manufacturer. Other DNA manipulations were done in accordance with standard protocol (31).

**Detection of the virulence plasmid.** To verify the presence of the virulence plasmid, two genes specific for this plasmid, \( \text{virR} \) (ORF4) and \( \text{aceA} \) (ORF12), were amplified by PCR using Taq DNA polymerase according to the manufacturer’s (Promega) instructions. A 200-bp fragment of the \( \text{virR} \) gene was amplified using 0.04F (5’-CGGACGAGTTTGACGTTGAT-3’) and 0.04R (5’-CAAGACGATTGCGGTGTCG-3’); a 200-bp fragment of \( \text{aceA} \) was amplified using 0.02F (5’-CAGTGACGCCTCAAGGCGA-3’) and 0.02R (5’-CAAGGCGCTGTAGTCGGAAC-3’).

**Construction of disruption vector pAP1.** Plasmid pAP1 contains an origin of replication derived from the vector pBLUESCRIPT and the \( \text{aceC}4 \) gene specifying resistance to ampicillin. This plasmid was digested with HindIII, treated with the Klenow fragment of DNA polymerase, and ligated to a 594-bp fragment of the \( \text{aceC}4 \) gene, which was amplified by PCR using the oligonucleotides 1015 (5’-CAAGCGCTACCAGCTCGAGAAGA-3’) and 1010 (5’-TCAGCGGCAAGCTCGAAG-3’). The resulting plasmid, pAP1, contains an internal fragment of the \( \text{aceC}4 \) gene.

**Electroporation of \( R. \ equi \).** \( R. \ equi \) was made electroporantent using the method described by Navas et al. (28); plasmids were introduced into \( R. \ equi \) by electroporation as previously described (24).

**Enzyme assays.** Cells were harvested in late-exponential-phase growth (optical density at 600 nm = 1). Cells were broken by three passages through a French pressure cell (Aminco) at 1,000 lbf/in\(^2\); followed by centrifugation (10 min, 14,000 \( 	imes g \), 4°C) to remove cell debris. Isocitrate lyase (EC 4.1.3.1) activity was determined at 37°C by measuring the formation of glyoxylate from phenylhydrazine in the presence of phenylhydrazine and isocitrate at 324 nm as described previously (7). The activity was determined according to the method of Bradford using bovine serum albumin as the standard (4).

**Macrophage infection.** Murine J774A.1 macrophages were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum, 2 mM glutamine, and 10 \( \mu \)g/mL gentamicin. Plates were seeded at a concentration of 1 \( \times 10^5 \) macrophages per well (final volume, 1 ml) and left for 48 h, feeding with growth medium after 24 h. Overnight LB broth cultures of \( R. \ equi \) 103P\(^+\), 103P\(^-\), and Ace-21 were grown to a density of 10\(^7\) CFU per ml. \( R. \ equi \) was washed twice with cation-free phosphate-buffered saline and resuspended in phagocytosis buffer (12). Monolayers were washed with warm DMEM, and phagocytosis buffer and normal mouse serum (5% [vol/vol]) were added. Macrophages were subsequently infected with wild-type and mutant strains at a multiplicity of infection of 15. Plates were incubated for 30 min at 37°C in 5% CO\(_2\). Monolayers were washed with warm DMEM, and phagocytosis buffer to remove unbound bacteria and incubated for a further 30 min to allow internalization of the bacteria. Phagocytosis buffer was replaced with DMEM supplemented with 10% (vol/vol) fetal calf serum, 2 mM glutamine, and 50 \( \mu \)g/mL gentamicin for 15 min. The medium was subsequently replaced with cation containing 10 \( \mu \)g/mL gentamicin. Medium was removed and monolayers washed with phosphate-buffered saline. Macrophages were then lysed with 0.5% (wt/vol) sodium dodecyl sulfate (SDS). Exposure to SDS was brief (20 s), allowing lysis of the macrophage but not affecting subsequent growth of \( R. \ equi \). \( R. \ equi \) was subsequently enumerated by plate counts on LB agar plates.

**Mouse infection.** Six groups of eight 6- to 8-week-old female CD1 mice were injected intravenously with 100 \( \mu \)l of \( 5 \times 10^6 \) CFU/ml of \( R. \ equi \) 103P\(^+\), 103P\(^-\), or Ace-21. The mice were euthanized 2 or 4 days after infection, and their livers were aseptically removed and ground in phosphate-buffered saline, pH 7.2. The suspended ground tissue was diluted in a 10-fold series and 50-\( \mu \)l aliquots plated on Trypticase soy agar (Difco). Bacterial colonies were counted after 48 h incubation at 37°C. Significant differences in bacterial numbers between mice infected with \( R. \ equi \) 103P\(^+\) and those infected with \( R. \ equi \) Ace-21 were determined by the two-tailed Student’s t test.

**Foal infection.** Infection and subsequent clinical assessment of foals was carried out as described previously (8). Briefly, four mixed-breed pony foals at 21 days of age were challenged with \( R. \ equi \) Ace-21 and four similarly aged foals were challenged with \( R. \ equi \) \( P^+\) by introducing 25 ml of cell suspension (5 \( \times 10^8\) cells/ml) into both main bronchi (total, 50 ml). Foals were clinically assessed based on daily complete physical examinations and twice-daily heart rate, respiratory rate, and temperature recordings. Foals were euthanized 14 days postinfection, and post-mortem examinations were performed to determine the lung-to-body-weight ratio, and to take lung samples to determine \( R. \ equi \) counts per gram of lung tissue from six preselected sites. Lesions, if any, in the lungs were examined for consistency with \( R. \ equi \) infection. The proportion of Ace-21 reverting to the wild type in foals infected with this mutant was determined by enumeration of \( R. \ equi \) on Trypticase soy agar plates with and without apramycin.

**Nucleotide sequence accession number.** The GenBank accession number of the sequence reported in this paper is AY738741.

## TABLE 1. Bacteria and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Characteristic(s)</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli DH5α</td>
<td>supE44 ΔaacC4Val169 [p888lacZΔM15] hsdS2 ΔrK1 endA1 gyrA96 thi-1 relA1</td>
<td>Stratagen e</td>
</tr>
<tr>
<td>R. equi 103P(^+)</td>
<td>Virulent foal isolate</td>
<td>6</td>
</tr>
<tr>
<td>R. equi 103P(^-)</td>
<td>Avirulent foal isolate lacking the virulence plasmid</td>
<td>6</td>
</tr>
<tr>
<td>R. equi Ace-21</td>
<td>aceC4 derivative of R. equi 103P(^+)</td>
<td>This study</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBluescript II KS</td>
<td>Ap(^+), shuttle vector</td>
<td>Stratagen e</td>
</tr>
<tr>
<td>pRE7</td>
<td>Km(^r), R. equi-Col shuttle vector</td>
<td>40</td>
</tr>
<tr>
<td>pAP1</td>
<td>Ap(^r), R. equi suicide vector</td>
<td>23</td>
</tr>
<tr>
<td>pICL1</td>
<td>Ap(^r), pBluescript containing a 1.8-kb HindIII-BamHI DNA fragment harbouring aceC4 of R. equi</td>
<td>19</td>
</tr>
<tr>
<td>pAP1</td>
<td>Ap(^r), Suicide vector for R. equi containing an internal 594-bp aceC4 fragment</td>
<td>This study</td>
</tr>
<tr>
<td>pKICL1</td>
<td>Km(^r), pRE7 containing a 1.8-kB HindIII-BamHI DNA fragment of pICL1 harbouring aceC4</td>
<td>This study</td>
</tr>
</tbody>
</table>
that *R. equi* 103 is more amenable to genetic manipulation than strain ATCC 33701 due to an approximately 100-fold-higher transformation frequency and reduced frequency of illegitimate recombination. We therefore chose strain 103 with which to construct an *aceA* mutant. The virulence plasmids of both strains have been sequenced and shown to be virtually identical, suggesting that the genomes of both strains are equally conserved (34). Prior to constructing an *aceA* mutant strain, we amplified a 1,126-bp DNA fragment containing an internal fragment of the *aceA* gene of *R. equi* 103. Nucleotide sequence analysis showed it to be identical to the gene of strain 33701, except for a neutral substitution in codon 47 encoding threonine.

**Construction of an isocitrate lyase-deficient *R. equi* mutant.** To construct an *R. equi* mutant lacking isocitrate lyase activity, the 2.9-kb plasmid pAIP1 was created, which contains the *aacC4* gene conferring apramycin resistance as a selectable marker and a 594-bp internal fragment of *aceA*. This plasmid was introduced into *R. equi* by electroporation, and the transformation mixture was subsequently plated onto LB agar plates containing apramycin. Because pAIP1 is unable to replicate in *R. equi*, apramycin-resistant colonies will contain pAIP1 integrated into the chromosome. To determine whether this was indeed the case, chromosomal DNA was isolated from two of the apramycin-resistant mutants, digested with BgIII, and analyzed by Southern hybridization using the 594-bp internal fragment as a probe. Since a BgIII restriction site does not occur within either the *aceA* gene or pAIP1, insertion of pAIP1 will increase the size of the BgIII fragment harboring *aceA* by 2.9 kb. Following hybridization of the *aceA* probe to BgIII-digested chromosomal DNA of two apramycin-resistant mutants (*R. equi* Ace-20 and Ace-21) and the wild-type strain, a single 8-kb hybridizing band was observed in the two mutants, whereas a 5-kb BgIII fragment was observed in the wild type strain (Fig. 1). To further confirm that pAIP1 had inserted correctly into the *aceA* gene, chromosomal DNA of one of the mutants (*R. equi* Ace-21) was digested with BgIII and subsequently ligated. The ligation mixture was used to transform *E. coli*, followed by selection for apramycin-resistant colonies, which contained an 8-kb plasmid harboring the disrupted *aceA* gene. Subsequent determination of the nucleotide sequence of the junction between the *aceA* gene and the point of insertion of pAIP1 showed that insertion had occurred via homologous recombination between *aceA* and the 594-bp internal *aceA* fragment (data not shown). Using virulence plasmid-specific primers amplifying the *virR* and *vapA* genes, it was shown that the *aceA* disruption mutant had retained the virulence plasmid (Fig. 1).

**Characterization of *R. equi* Ace-21.** We previously determined that *R. equi* contains high activities of isocitrate lyase following growth on acetate- or lactate-containing minimal medium (19). Isocitrate lyase activity is essential for growth on acetate, since it allows assimilation of this C₂ compound via the glyoxylate bypass. However, unless lactate is metabolized using lactate oxidase, which converts lactate to CO₂ and acetate, isocitrate lyase is not required for lactate metabolism. As expected, *R. equi* Ace-21 failed to grow on acetate, whereas growth on lactate- and succinate-containing minimal media was comparable to that of the wild type. The activity of isocitrate lyase in cell extracts of wild-type and mutant strains following growth on lactate was 586 nmol per min per mg of protein for the wild type, whereas this enzyme activity was absent in *R. equi* Ace-21. These results demonstrate that although *aceA* is expressed during growth on lactate, it is dispensable for the metabolism of this substrate. In order to rule out that the failure of *R. equi* Ace-21 to grow on acetate...
minimal medium was due to a mutation other than disruption of the aceA gene, plasmid pKICLA harboring an intact aceA gene and its promoter was introduced in R. equi Ace-21. In contrast to the mutant strain, a strain harboring this plasmid was able to use acetate as the sole source of carbon.

R. equi Ace-21 is unable to proliferate in macrophages. Intracellular survival and proliferation of R. equi Ace-21 in the murine macrophage-like cell line (J774A.1) was compared to those of the wild-type strain and its avirulent plasmid-free derivative. To this end, macrophages were incubated with R. equi and allowed to internalize, after which gentamicin was added to kill extracellular bacteria. Intracellular bacteria were enumerated by plate counts following macrophage lysis. The data represent the averages for two independent experiments. Plate counts were carried out in duplicate. Values are expressed as means ± standard deviations (error bars).

R. equi Ace-21 is attenuated in mice. Although immunocompetent mice eventually clear R. equi, the increase in R. equi numbers in the liver and spleen 2 to 4 days after intravenous injection provides a good indication of R. equi virulence in vivo (8). To assess the virulence of R. equi Ace-21, mice were intravenously injected with this strain, and the number of R. equi Ace-21 bacteria in the liver was compared to those in mice infected with isogenic virulent (103 PFU/mouse) or avirulent (103 PFU/mouse) R. equi. The avirulent plasmid-free strain was completely cleared 2 days after infection. In contrast, both the virulent and isocitrate lyase-deficient strain could be detected in the livers of mice 2 and 4 days following infection. However, there was a significant difference (day 2, P = 9.5 × 10−5; day 4, P = 9.6 × 10−5) in bacterial load between the two strains (Fig. 3), showing that R. equi Ace-21 is partially attenuated in mice.

In vivo infection of foals. The previous experiments using macrophages and mice to assess virulence of R. equi Ace-21 indicated that R. equi Ace-21 is attenuated. However, unlike foals, mice are naturally resistant to R. equi infections, and the route of infection in mice by intravenous injection is different from that in foals, which are challenged via the respiratory route. The outcome of the macrophage and mouse studies may therefore not necessarily reflect virulence of R. equi Ace-21 in foals. To evaluate virulence of R. equi Ace-21 in its native host, 3-week-old pony foals were intrabronchially infected with R. equi Ace-21, and results were compared to those for foals infected with R. equi 103 PFU/mouse. The temperature and heart and respiratory rates of foals infected with R. equi 103 PFU/mouse started to increase 9 days postinfection, whereas these parameters remained unaltered in foals infected with R. equi Ace-21 (Fig. 4). The foals were subjected to a post-mortem analysis immediately following euthanasia. The lungs from foals infected with virulent R. equi displayed severe lesions of suppurative to pyogranulomatous bronchopneumonia. In contrast, of the four foals infected with R. equi Ace-21, none showed any signs of granulomatous or other pneumonia in the lung tissue upon post-mortem examination. The lungs were completely normal both on gross visual inspection and on palpation. The lung weight/body weight ratio of control foals infected with R. equi 103 PFU/mouse was 3.83% ± 1.18%, whereas this ratio was 1.15% ± 0.06% in foals infected with the isocitrate lyase-deficient strain. These values compare to 4.58% ± 0.96% for infected historical
control foals and to 1.13% ± 0.07% observed previously for noninfected historical control foals (8). The mean number of *R. equi* bacteria per gram of tissue in Ace-21 infected foals was $10^{0.85} \pm 0.59$ per gram of lung tissue, compared to $10^{0.61} \pm 0.30$ for 103P*+-infected foals.

**DISCUSSION**

This paper focused on the role of the glyoxylate bypass in virulence of *R. equi* by analyzing virulence of an isocitrate lyase-deficient mutant. The glyoxylate bypass, which consists of the combined activities of isocitrate lyase and malate synthase, circumvents two decarboxylation reactions of the citric acid cycle (20). Without this pathway, assimilation of carbon substrates, which enter the central metabolic pathways at the level of acetyl-CoA, would not be possible, since they would exclusively be converted to CO$_2$. Examples of two carbon compounds are ethanol and acetate, but also fatty acids, which are metabolized to acetyl-CoA by β-oxidation.

Virulence of the isocitrate lyase mutant was assessed in three ways: macrophage survival, clearance in mice after intravenous infection, and infection in foals. The data show that the three different methods of assessing virulence gave similar results. A comparison of the three approaches has not previously been done, although there appears to be an assumption that these are correlated (8, 18). Survival in macrophages (Fig. 2) and in mice (Fig. 3) measure the ability of the organism to survive the innate immunity provided by macrophages. The observed attenuation of *R. equi* Ace-21 in these models was supported by infection studies with 3-week-old foals, in which the *aceA* mutant was found to be completely attenuated, since clinical and pathological findings were similar to those associated with infection of foals with a virulence plasmid-negative and therefore avirulent isolate (8, 15).

The observed attenuation of isocitrate lyase-deficient *R. equi* is in agreement with the results from previous experiments, which showed that isocitrate lyase-negative *M. tuberculosis* and *C. albicans* strains are attenuated (21, 25). The dependence on the glyoxylate cycle for virulence strongly indicates that these intracellular pathogens utilize fatty acids derived from macrophage membrane lipids as a source of carbon. However, *R. equi* appears to be more dependent on the glyoxylate bypass than *M. tuberculosis*. In the mouse model, isocitrate lyase-deficient *M. tuberculosis* initially behaved like the wild-type strain, leading to comparable bacterial burdens in the lungs of infected mice 2 weeks following infection. Only in the subsequent weeks did the difference in virulence between the isocitrate lyase-deficient and wild strains become apparent, with the former being progressively cleared whereas the latter persisted. The lack of a functional isocitrate lyase did not seriously affect *M. tuberculosis* survival in resting macrophages, since only in activated macrophages was there a dramatic difference in survival between wild-type and mutant strains (21, 25). In contrast, the isocitrate lyase-deficient *R. equi* strain failed to induce disease in foals, and although *R. equi* could be recovered from lungs of foals infected with *R. equi* Ace-21, the number of bacteria was about six orders of magnitude lower than usually observed with the wild-type strain (9, 14). Furthermore, the isocitrate lyase-deficient strain failed to proliferate in resting macrophages, whereas the wild-type strain grew with a doubling time of approximately 8 h. It thus appears that *R. equi* is less versatile than *M. tuberculosis* with regard to the number of host-derived carbon sources it can metabolize.

*R. equi* is the latest addition to a growing number of pathogenic microorganisms that require the glyoxylate bypass for virulence (17, 21, 25, 38, 39). The dependence on this pathway for virulence appears to be a unifying factor; however, the underlying reasons for this requirement differ. *M. tuberculosis*, *C. albicans*, and *R. equi* are intracellular pathogens of mammalian phagocytic cells, which most likely require the glyoxylate cycle for assimilation of macrophage lipids (21, 25). Virulence of the plant pathogen *R. fascians* is dependent on malate synthase, which may be required for the assimilation of

**FIG. 4.** Heart rates (A), temperatures (B), and respiratory rates (C) of foals infected with *R. equi* P*+* (●) or *R. equi* Ace-21 (△). Values are shown as the means ± standard deviations (error bars) within each group.
products of photorespiration, such as glycolate (38). The fungus *Magnaporthe grisea* appears to employ the glyoxylate bypass to metabolize fungal lipids for turgor generation and appressorium formation prior to infection (39).

The *aceA* gene is the second gene after *vapA* (18) to be shown to be essential for virulence of *R. equi*. In addition to being essential for assimilation of fatty acids, isocitrate lyase is also required for assimilation of acetate, which is present in significant amounts in the large intestine of horses. *R. equi* grows well on this substrate, and it has been shown previously that acetate stimulates growth of this pathogen in dung (16). It is therefore clear that although isocitrate lyase is not a true virulence factor like VapA, it plays a critical role in growth and survival of *R. equi* both within and outside the host environment.

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