Microbiology (2002), **148**, 793–798 Printed in Great Britain

Isocitrate lyase of the facultative intracellular pathogen *Rhodococcus equi*

Bridget G. Kelly, Daniel M. Wall, Clara A. Boland and Wim G. Meijer

Author for correspondence: Wim G. Meijer. Tel: +353 1 716 1364. Fax: +353 1 716 1183.
e-mail: wim.meijer@ucd.ie

Department of Industrial Microbiology, Conway Institute of Biomolecular and Biomedical Sciences, University College Dublin, Dublin 4, and Dublin Molecular Medicine Centre, Dublin, Ireland

Isocitrate lyase is the first enzyme of the glyoxylate shunt which is required for the assimilation of fatty acids and acetate. The intracellular pathogen *Rhodococcus equi* contains high activities of this enzyme following growth on acetate and lactate, indicating that it plays an important role in the metabolism of these substrates. The gene encoding isocitrate lyase (*aceA*) was cloned and sequenced. It specifies a 46 846 Da protein, which was shown to be functional by expressing it in *Escherichia coli*. A gene similar to *fadB*, encoding 3-hydroxyacyl-CoA dehydrogenase, was located 90 bp downstream from *aceA*. Northern hybridization and RT-PCR experiments showed that *aceA* and *fadB* are cotranscribed into a 2·8 kb transcript. A smaller 1·6 kb *aceA* transcript was also observed which was 2·5-fold more abundant than the *aceA*-*fadB* transcript. It is proposed that a stable hairpin structure with a free energy (Δ*G*) of −28·5 kcal mol^{-1} and located in the 90 bp *aceA*-*fadB* intergenic region is involved in stabilizing the *aceA* transcript.

Keywords: *aceA*, *fadB*, lactate and acetate metabolism, glyoxylate shunt, mRNA processing

INTRODUCTION

Rhodococcus equi is a major pathogen of young foals, causing severe pyogranulomatous bronchopneumonia. In addition, *R. equi* is increasingly recognized as an opportunistic pathogen of humans, in particular of immunocompromised patients (Mosser & Hondalus, 1996). *R. equi* is ubiquitous in soils, especially if these are enriched with faeces. It has been shown that components contained in horse dung, in particular acetate and propionate, stimulate growth of *R. equi* in this environment (Hughes & Sulaiman, 1987). The primary route of infection is via inhalation of dust particles emanating from soils in which this nocardiaform actinomycete rapidly proliferates.

Virulence of *R. equi* is dependent on an 81 kb plasmid, indicating that this plasmid encodes virulence factors allowing the pathogen to establish itself in the host. Although the plasmid has been completely sequenced (Takai *et al.*, 2000), it is still unknown what role the encoded proteins play in virulence. Although it has been firmly established that the virulence plasmid is essential for infection of foals (Takai *et al.*, 1993; Hondalus & Mosser, 1994), it is clear that chromosomally encoded factors, such as regulatory genes (Boland & Meijer, 2000), and metabolic pathways (Linder & Bernheimer, 1997; Navas *et al.*, 2001) are equally important in allowing the pathogen to thrive within the host.

R. equi primarily infects alveolar macrophages of young foals and immunocompromised humans. Following uptake by macrophages, virulent *R. equi* strains grow rapidly in the phagolysosome (Hondalus & Mosser, 1994). At present it is poorly understood what sources of carbon, nitrogen and other essential nutrients are used during intracellular growth of this and other intracellular pathogens. Obvious sources of carbon are membrane cholesterol (Navas *et al.*, 2001) and lipids of the phagolysosome within which the pathogen resides. Lipids are generally dissimilated via beta-oxidation, resulting in the formation of acetyl-CoA, which is further metabolized via the tricarboxylic acid (TCA) cycle.

To assimilate acetate and lipids via acetyl-CoA, the two oxidative steps of the TCA cycle have to be bypassed. In most bacteria this is accomplished by the glyoxylate shunt, in which the subsequent activities of isocitrate lyase and malate synthase convert isocitrate and acetyl-CoA to succinate and malate. The relative flux of carbon through the assimilatory glyoxylate shunt or the dis-

The GenBank accession number for the sequence reported in this paper is AY044917.

similatory TCA cycle is controlled by the relative activities of isocitrate lyase and isocitrate dehydrogenase, which compete for their common substrate isocitrate (Nimmo *et al.*, 1987). It was recently shown that isocitrate lyase mutants of *Mycobacterium tuberculosis* and *Candida albicans* are strongly reduced in their ability to persist in mice or inflammatory macrophages. This emphasizes the importance of the glyoxylate shunt in long-term persistence in the host (McKinney *et al.*, 2000; Lorenz & Fink, 2001) and supports the notion that membrane lipids may serve as a source of carbon for these and other intracellular pathogens.

The use of acetate and the potential use of membrane lipids by *R. equi* as carbon sources in soil and within macrophages, respectively, indicates that the glyoxylate shunt is important for the proliferation of both free-living and intracellularly located *R. equi*. To date virtually nothing is known regarding the glyoxylate shunt of *R. equi*. This paper shows that isocitrate lyase is maximally expressed following growth on acetate and lactate. The gene encoding isocitrate lyase was cloned and the transcriptional organization of this gene and *fadB*, encoding 3-hydroxyacyl-CoA dehydrogenase, was determined.

METHODS

Bacterial strains and plasmids. *R. equi* ATCC 33701 and *Rhodococcus fascians* D188 (LMG 3605) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and the Belgian Co-ordinated Collections of Microorganisms (BCCM/LMG, Collection of the Laboratorium voor Microbiologie en Microbiele Genetica, Gent, Belgium), respectively. *Escherichia coli* DH5α (*supE44* Δ*lac*U169 [ϕ80*lacZ*ΔM15] *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*) was used as a host for the cloning vector pBluescript II KS and its derivatives.

Media and growth conditions. *E. coli*, *R. fascians* and *R. equi* strains were grown on Luria–Bertani (LB) medium at 37 °C (Sambrook & Russell, 2000). Minimal *R. equi* medium consisted of (g l^{-1}): K_2HPO_4, 5·0; $NaH_2PO_4.H_2O$, 1·5; $MgSO_4.7H_2O$, 0·2; $(NH_4)_2SO_4$, 1·0. Trace elements solution (0·2 ml; Vishniac & Santer, 1957) and thiamine (0·1 mM) were added per litre of medium. Minimal medium was supplemented with acetate (20 mM), succinate (10 mM), lactate (20 mM) or pyruvate (20 mM). When appropriate, the following supplements were added: ampicillin, 50 μg ml^{-1}; X-Gal, 20 μg ml^{-1}; IPTG, 0·1 mM. Agar was added for solid media (1·5 %, w/v).

DNA manipulations. Plasmid DNA was isolated with the alkaline lysis method (Birnboim & Doly, 1979) or by using the Wizard Plus SV miniprep (Promega). Chromosomal DNA was isolated as described by Nagy *et al.* (1995). 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 2000 automatic sequencer; nucleotide sequence data were compiled using the Staden package (Staden *et al.*, 2000).

Southern hybridization. Restriction digests of chromosomal DNA of *R. equi* were transferred to a positively charged nylon membrane as recommended by the manufacturer (Roche) following agarose gel electrophoresis. A 269 bp fragment representing an internal fragment of the isocitrate lyase gene of *R. fascians* (accession no. Z29367) was amplified by PCR with *Pwo* polymerase (Roche) using the oligonucleotides Rficfor (5′-AGGCTTCTTCGGTGTCAAGA-3′) and Rficrev (5′-TGAACTGGAACTTGAAGCCC-3′). The reaction mixture was incubated at 94 °C for 5 min and was subsequently subjected to 30 cycles of 94 °C for 30 s, 55 °C for 45 s and 72 °C for 1 min, followed by an incubation at 72 °C for 5 min. The fragment was labelled with digoxigenin 11-dUTP using the DIG High Prime Kit (Roche). Prehybridization, hybridization and detection of the labelled probe were done according to the manufacturer's recommendations.

RNA isolation. Cells were harvested by centrifugation at 20000 ***g*** for 5 min; pellets were resuspended in 100 μl TES (10 mM Tris/HCl, pH 8, 1 mM EDTA, 1 %, w/v, SDS) and incubated at 70 °C. The cell suspension was subsequently subjected to five cycles of incubation at 70 °C, followed by freezing in liquid nitrogen. RNA was isolated using the Promega SV RNA isolation kit according to the manufacturer's instructions.

Northern hybridization. Following electrophoresis on a denaturing formaldehyde gel (Sambrook & Russell, 2000), RNA was transferred to a positively charged membrane according to the manufacturer's instructions (Roche). An internal fragment of the isocitrate lyase gene of *R. equi* was labelled by PCR amplification using the oligonucleotides Icl5 (5′-CAACGTCTACGAGCTGCAGA-3′) and Icl10 (5′-TCGAACATGCCGTAGTTGAG-3′) in the presence of 0·2 mM dATP, dCTP and dGTP; 0·13 mM dTTP and 0·07 mM digoxigenin 11-dUTP, using *Taq* polymerase according to the manufacturer's instructions (Promega). The reaction mixture was incubated at 94 °C for 5 min and was subsequently subjected to 30 cycles of 94 °C for 30 s, 50 °C for 1 min and 74 °C for 1 min, followed by an incubation at 74 °C for 7 min. Prehybridization, hybridization and chemiluminescent detection of the labelled probe using DIG Easy Hyb and CDP-Star (Roche) were done according to the manufacturer's recommendations. The relative abundance of transcripts was determined using a Fluor-S Max Multiimager System (Bio-Rad) and the Quantity One software package (Bio-Rad).

RT-PCR. RT-PCR was carried out with the Promega Access RT-PCR System as per manufacturer's instructions using mRNA isolated from acetate-grown *R. equi*. RNA corresponding to a 411 nt fragment was amplified using the oligonucleotides Icl24 (5′-ACCTGGCGCACGGCTACGCCC-3′) and Icf2 (5′-GCTCGTACACCAGCACGTC-3′). The reaction mixture was incubated at 48 °C for 45 min and 94 °C for 5 min. It was subsequently subjected to 40 cycles at 94 °C for 30 s, 55 °C for 45 s and 68 °C for 1 min, followed by an incubation at 68 °C for 7 min. RNase-treated samples and reaction mixtures in which the AMV reverse transcriptase was omitted were used as control reactions.

Enzyme assays. Cells were harvested at late exponential growth phase ($OD_{430} = 1$). Isocitrate lyase (EC 4.1.3.1) activity was determined at 37 °C in cell extracts (Dijkhuizen *et al.*, 1978) by measuring the formation of glyoxylate phenylhydrazone in the presence of phenylhydrazine and isocitrate at 324 nm as described by Dixon & Kornberg (1959). Isocitrate dehydrogenase (EC 1.1.1.42) was determined at 37 °C by measuring the reduction of NADP at 340 nm (Levering & Dijkhuizen, 1985). Protein was determined according to Bradford (1976) using bovine serum albumin as standard.

RESULTS

Isocitrate lyase and isocitrate dehydrogenase activities during growth on various carbon sources

Isocitrate is present at a branching point between assimilation via the glyoxylate cycle and dissimilation via the TCA cycle. To demonstrate the presence of isocitrate lyase and to determine the relative contributions of each pathway to the flux of carbon through these pathways during growth on various carbon sources, the activities of isocitrate lyase and isocitrate dehydrogenase were examined following growth on acetate, lactate and succinate. The growth rate of *R. equi* growing on lactate was 0·27 ± 0·01 and 0·12 ± 0·01 h^{-1} during growth on succinate and acetate; growth was completely dependent on the presence of thiamine. As expected, high levels of isocitrate lyase activities were observed following growth on acetate, emphasizing the importance of this pathway in the assimilation of carbon derived from this substrate. Surprisingly, the activity of this enzyme was almost equally high following growth on lactate, suggesting that the glyoxylate cycle may play an important role in lactate metabolism (Table 1). The ratio of isocitrate lyase and isocitrate dehydrogenase activities following growth on these substrates was approximately one, indicating that there is a substantial flux of carbon through the assimilatory glyoxylate pathway. In contrast, this ratio was very low in succinate-grown cells, indicating that the glyoxylate shunt does not play a significant role in succinate metabolism (Table 1).

Cloning of the isocitrate lyase gene *aceA*

To determine whether *R. equi* harbours an isocitrate lyase gene similar to that of *R. fascians* (Vereecke *et al.*, 1994), a 269 bp DNA fragment was amplified by PCR from the *R. fascians* genome. Southern hybridization of chromosomal DNA of *R. equi* using the 269 bp PCR product as heterologous probe showed that the *aceA* gene was present on a 5·5 kb *Bam*HI fragment (data not shown). Only one hybridizing DNA fragment was observed, indicating that *R. equi* harbours a single isocitrate lyase gene homologous to the *R. fascians* gene. A mini-library of *Bam*HI fragments, ranging in size between 4 and 6 kb, was subsequently constructed in the *Bam*HI site of pBluescript KS II, transformed into *E. coli* DH5α and screened with the heterologous *aceA* probe. This led to the identification of a single colony which contained a plasmid (pICL1) with a 5·5 kb *Bam*HI insert hybridizing to the *aceA* probe. Further subcloning reduced the size of the DNA fragment containing the sequences hybridizing to the *aceA* probe to a 1·8 kb *Hin*dIII–*Bam*HI fragment (pICL11). The *aceA* gene was localized on this fragment by PCR using Rficfor and Rficrev in conjunction with oligonucleotides complementary to *lacZ*, in which the presence and length of the PCR product indicates the position and direction of transcription of *aceA* on pICL11 (Fig. 1).

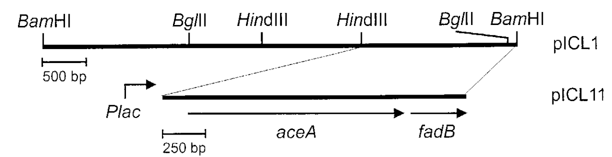

Fig. 1. Restriction map of the 5·5 kb *Bam*HI insert of pICL1 and pICL11. Arrows indicate the position and direction of transcription of the genes encoding isocitrate lyase (*aceA*) and 3-hydroxyacyl-CoA dehydrogenase (*fadB*). The position of the *lac* promoter of pBluescript II KS is indicated.

To determine whether the *aceA* gene was functional, *E. coli*(pICL11) was grown in LB medium supplemented with 1 mM IPTG to induce transcription from the *lac* promoter. The isocitrate lyase activity in *E. coli*(pICL11) was sixfold higher [138 ± 5 nmol min^{-1} (mg protein)$^{-1}$] than in *E. coli* cells harbouring pBluescript II KS [22 ± 3 nmol min^{-1} (mg protein)$^{-1}$], showing that the *aceA* gene present on pICL11 encodes an active isocitrate lyase.

Nucleotide sequence of the *aceA* gene

Following nucleotide sequencing, one ORF preceded by a plausible ribosome-binding site was identified, which could encode a protein with a molecular mass of 46 846 Da. Since the protein sequence specified by this ORF was 93 % identical to that of *R. fascians* (Vereecke *et al.*, 1994), the ORF was identified as *aceA*, encoding isocitrate lyase. The 5′ end of a second ORF which was preceded by a ribosome-binding site and initiated with a GTG codon was identified 90 bp downstream of the

Table 1. Activities and ratios of isocitrate lyase and isocitrate dehydrogenase in *R. equi*, following growth on various carbon sources

The data represent the means of three independent experiments. The values differ from the means by < 10 %.

Growth substrate	Isocitrate lyase activity [nmol min^{-1} (mg protein)$^{-1}$]	Isocitrate dehydrogenase activity [nmol min^{-1} (mg protein)$^{-1}$]	Ratio
Acetate	1115	942	1·2
Lactate	855	945	0·9
Succinate	17	307	0·06

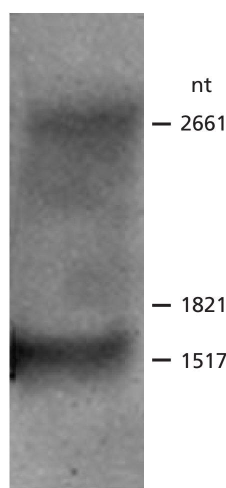

Fig. 2. Northern hybridization of an internal *aceA* fragment to mRNA isolated from acetate-grown *R. equi*.

aceA gene. The protein specified by this ORF was highly similar to FadB2, a 3-hydroxyacyl-CoA dehydrogenase from *M. tuberculosis* (66% identity). This ORF was therefore tentatively identified as *fadB*. Analysis of the 90 bp *aceA-fadB* intergenic region revealed the presence of a 17 bp inverted repeat, which may form a stable hairpin structure with a free energy (ΔG) of $-28{\cdot}5$ kcal mol^{-1}. A stretch of thymidines downstream from the hairpin structure, characteristic for ρ-independent transcriptional terminators (Platt, 1986), was not present, indicating that this structure does not function as such.

aceA and *fadB* are cotranscribed

The small intergenic region between *aceA* and *fadB* strongly suggested that these genes are transcribed into a single transcript. To determine the transcriptional organization of this locus, mRNA was isolated from acetate-grown *R. equi*, during which growth the *aceA* gene is highly expressed. Northern hybridization, using an internal *aceA* fragment as probe, revealed two *aceA* transcripts of 2·8 and 1·6 kb (Fig. 2). Image analysis showed that the smaller transcript was 2·5 times more abundant than the larger transcript. The *aceA* gene is 1289 bp, whereas *fadB* genes are typically 858 bp. This indicates that the 2·8 kb transcript is at least bicistronic, encompassing both *aceA* and *fadB*. This was further examined by subjecting mRNA isolated from acetate-grown cells to RT-PCR using oligonucleotides complementary to *aceA* and *fadB*. A 411 bp product was observed encompassing the 3′ end of *aceA* and the 5′ end of *fadB* (data not shown), showing that these genes are cotranscribed.

DISCUSSION

The glyoxylate shunt is essential for growth on acetate and lipids. The former has been identified as an important source of carbon for free-living *R. equi* in soil (Hughes & Sulaiman, 1987); the latter is likely to support growth of *R. equi* in the phagolysosome. The main function of this pathway is to bypass two oxidative steps of the TCA cycle, thus preventing the loss of acetyl-CoA-derived carbon as CO_2. The first reaction of this pathway is catalysed by isocitrate lyase, an enzyme converting isocitrate to glyoxylate and succinate. In Gram-negative bacteria the synthesis of this enzyme is usually strictly regulated; isocitrate lyase activities are only observed following growth on acetate or fatty acids when operation of the glyoxylate shunt is required to allow assimilation of acetyl-CoA (see for example, Kornberg, 1966; Meijer & Dijkhuizen, 1988; Green *et al*., 1998). Expression of isocitrate lyase activity in *R. equi* is not as strict. During growth on succinate significant activities of isocitrate lyase were present, which was also noted for the mycolic-acid-containing actinomycetes *Corynebacterium glutamicum*, *M. tuberculosis* and *Mycobacterium avium* (Wendisch *et al*., 1997; Höner zu Bentrup *et al*., 1999).

Surprisingly, the levels of isocitrate lyase and the ratio of isocitrate lyase and isocitrate dehydrogenase during growth on lactate were the same as during growth on acetate. In most bacteria lactate is metabolized to pyruvate by lactate dehydrogenase. Conversion of pyruvate to oxaloacetate by pyruvate carboxylase, or conversion of pyruvate to phosphoenolpyruvate followed by the formation of oxaloacetate by phosphoenolpyruvate carboxylase serve as anapleurotic reactions. These and other anapleurotic enzymes employed during growth on lactate enable the bacterium to assimilate carbon from lactate; isocitrate lyase is therefore not required for growth on lactate by bacteria employing lactate dehydrogenase (Meijer & Dijkhuizen, 1988). An explanation for the unexpectedly high activity of isocitrate lyase during growth on lactate could be that *R. equi* employs a lactate monooxygenase instead of lactate dehydrogenase. This enzyme, which is present in the closely related *Mycobacterium smegmatis*, converts lactate into acetate, carbon dioxide and water (Giegel *et al*., 1990). If *R. equi* indeed employs lactate monooxygenase to metabolize lactate, then the glyoxylate shunt is required to assimilate acetate produced by this enzyme. The role of lactate monooxygenase in lactate metabolism of *R. equi* is currently under investigation in this laboratory.

Nucleotide sequencing showed that *aceA* is clustered with a gene similar to *fadB*, encoding 3-hydroxyacyl-CoA dehydrogenase, an enzyme participating in beta-oxidation of fatty acids. Analysis of *aceA* transcription using Northern hybridization showed the presence of two *aceA* mRNA species of 2·8 and 1·6 kb. The former transcript is sufficiently large to accommodate both *aceA* and *fadB*, which was confirmed by RT-PCR using oligonucleotides complementary to *aceA* and *fadB*. The presence of a smaller *aceA* mRNA, 2·5-fold more abundant than the *aceA-fadB* transcript, indicates that the *aceA-fadB* transcript is processed. The intergenic region between *aceA* and *fadB* contains a 17 bp inverted repeat capable of forming a stable hairpin structure.

These structures are generally involved in stabilizing the upstream transcript by protecting it from 3′-5′ exonuclease attack (Mclaren *et al.*, 1991). The apparent *aceA-fadB* processing and the greater abundance of the *aceA* transcript indicates that this structure fulfils this role in *R. equi*.

The *aceA* and *fadB* genes are arranged in an identical manner in *M. tuberculosis* and, since they are separated by only 81 bp, are likely to form an operon. Genomic sequencing of the *Streptomyces coelicolor* genome (accession no. AL596102) showed that in this bacterium *aceA* is also clustered with *fadB*. However, in contrast to the mycolic-acid-containing actinomycetes, *S. coelicolor* harbours a malate synthase gene (*aceB2*) 100 bp downstream of *aceA*, followed by *fadB*. The small spacing between *aceA* and *aceB2* and the fact that the stop codon of *aceB2* and the start codon of *fadB* overlap, indicate that these three genes are cotranscribed as an operon.

Interestingly, in these three actinomycetes the *aceA* gene is separated from *fadB* (*R. equi* and *M. tuberculosis*) or *aceB2-fadB* (*S. coelicolor*) by an intergenic region of 81–100 bp. Analysis of the 81 bp intergenic region between *aceA* and *fadB2* and the 100 bp intergenic region between *aceA* and *aceB2* revealed the presence of stable 17 bp ($\Delta G = -24{\cdot}5$ kcal mol^{-1}) and 15 bp ($\Delta G = -30{\cdot}1$ kcal mol^{-1}) hairpin structures in *M. tuberculosis* and *S. coelicolor*, respectively. This strongly suggests that the putative *aceA-fadB2* and *aceA-aceB2-fadB* transcripts in these bacteria are processed in a similar manner as in *R. equi*, leading to a stable *aceA* transcript. Isocitrate lyase is the first enzyme of the glyoxylate shunt and competes with isocitrate dehydrogenase for isocitrate. High isocitrate lyase activities are therefore required to create a sufficient flux of carbon through this pathway. In actinomycetes this seems to be accomplished at least in part by a stable *aceA* transcript which would allow high expression levels of this gene. Our current studies aim to further characterize mRNA processing of the *aceA-fadB* transcript of *R. equi*.

ACKNOWLEDGEMENTS

This study was supported by a grant from the Health Research Board (133/99).

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Received 13 August 2001; revised 16 October 2001; accepted 29 October 2001.