The methylcitric acid pathway in *Ralstonia eutropha*: new genes identified involved in propionate metabolism

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From *Ralstonia eutropha* HF39 null-allele mutants were created by Tn5 mutagenesis and by homologous recombination which were impaired in growth on propionic acid and levulinic acid. From the molecular, physiological and enzymic analysis of these mutants it was concluded that in this bacterium propionic acid is metabolized via the methylcitric acid pathway. The genes encoding enzymes of this pathway are organized in a cluster in the order *prpR*, *prpB*, *prpC*, *acnM*, ORF5 and *prpD*, with *prpR* transcribed divergently from the other genes. (i) *prpC* encodes a 2-methylcitric acid synthase (42 720 Da) as shown by the measurement of the respective enzyme activity, complementation of a *prpC* mutant of *Salmonella enterica* serovar Typhimurium and high sequence similarity. (ii) For the translational product of *acnM* the function of a 2-methyl-*cis*-aconitic acid hydratase (94 726 Da) is proposed. This protein and also the ORF5 translational product are essential for growth on propionic acid, as revealed by the propionic-acid-negative phenotype of Tn5-insertion mutants, and are required for the conversion of 2-methylcitric acid into 2-methylisocitric acid as shown by the accumulation of the latter, which could be purified as its calcium salt from the supernatants of these mutants. In contrast, inactivation of *prpD* did not block the ability of the cell to use propionic acid as carbon and energy source, as shown by the propionic acid phenotype of a null-allele mutant. It is therefore unlikely that *prpD* from *R. eutropha* encodes a 2-methyl-*cis*-aconitic acid dehydratase as proposed recently for the homologous *prpD* gene from *S. enterica*. (iii) The translational product of *prpB* encodes 2-methylisocitric acid lyase (32 314 Da) as revealed by measurement of the respective enzyme activity and by demonstrating accumulation of methylisocitric acid in the supernatant of a *prpB* null-allele mutant. (iv) The expression of *prpC* and probably also of the other enzymes is regulated and is induced during cultivation on propionic acid or levulinic acid. The putative translational product of *prpR* (70 895 Da) exhibited high similarities to PrpR of *Escherichia coli* and *S. enterica*, and might represent a transcriptional activator of the sigma-54 family involved in the regulation of the other *prp* genes. Since the *prp* locus of *R. eutropha* was very different from those of *E. coli* and *S. enterica*, an extensive comparison of *prp* loci available from databases and literature was done, revealing two different classes of *prp* loci.

Keywords: propionyl-CoA, *Ralstonia eutropha*, 2-methylcitric acid cycle, propionic acid catabolism, methylcitric acid synthase

Abbreviation: DIG, digoxigenin.

The GenBank accession numbers for the nucleotide sequences of the *prp* gene cluster are AF325554 and AF331923.

INTRODUCTION

The Gram-negative bacterium *Ralstonia eutropha* HF39 grows on propionate and on several other substrates as sole carbon and energy source, which are catabolized via propionyl-CoA. In prokaryotes different strategies are used for the catabolism of this short-chain fatty acid, e.g. the acrylate pathway, the methylmalonyl-CoA pathway or the malonic semialdehyde-CoA pathway (Vagelos, 1959). In addition, the cyclic 2-methylcitric acid pathway has been identified as a new strategy for the breakdown of propionate in the yeast *Saccharomyces cerevisiae* and in filamentous fungi (Miyakoshi *et al.*, 1987; Pronk *et al.*, 1994) as well as in the Gram-negative bacteria *Salmonella enterica* serovar Typhimurium and *Escherichia coli* (Horswill & Escalante-Semerena, 1997; Textor *et al.*, 1997). In the enterobacteria the genes encoding the enzymes of this cycle are organized in a cluster (*prpBCDE*) (Horswill & Escalante-Semerena, 1997; Blattner *et al.*, 1997). The *prpE* gene product catalyses the activation of propionate to propionyl-CoA (Horswill & Escalante-Semerena, 1999a). Propionyl-CoA and oxaloacetate are then condensed to 2-methylcitric acid in a Claisen ester condensation which is catalysed by the methylcitric acid synthase encoded by *prpC* and which is the first characteristic step of this pathway. 2-Methylcitric acid is then dehydrated to 2-methyl-*cis*-aconitic acid and subsequently hydrated to 2-methylisocitric acid. This latter hydration is catalysed by a 2-methylisocitric acid dehydratase in *Yarrowia lipolytica* (Aoki *et al.*, 1995). 2-Methylisocitric acid is cleaved into pyruvate plus succinate by the activity of the *prpB* gene product, which shows similarities to isocitric acid lyases (Horswill & Escalante-Semerena, 1999b). *prpR* is a potential transcriptional activator of the *prp* genes and exhibits homologies to members of the RpoN (σ^{54}) activator family.

During the investigation of Tn5-induced mutants of *R. eutropha* HF39 with defects in the catabolism of levulinic acid (4-oxopentanoic acid) (Valentin *et al.*, 1992; Gorenflo *et al.*, 1998), we identified a gene cluster, the gene products of which constitute the methylcitric acid cycle in this bacterium. In this study, we present molecular and biochemical data of the methylcitric acid pathway in *R. eutropha* HF39 and compare the organization of its methylcitric acid cycle genes with those of other bacteria.

METHODS

Strains, media and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. Cells of *E. coli* were cultivated at 37 °C in Luria-Bertani (LB) medium (Sambrook *et al.*, 1989). Cells of *R. eutropha* HF39 were grown at 30 °C either in nutrient broth (NB) medium (0.8%, w/v) or in mineral salts medium (MM) (Schlegel *et al.*, 1961) supplemented with filter-sterilized carbon sources as indicated in the text. Solid media contained 1.5% (w/v) purified agar. For the selection of Tn5-insertion or null-allele mutants obtained by homologous recombination of *R. eutropha* HF39, kanamycin (160 µg ml⁻¹) and streptomycin (500 µg ml⁻¹) were

added to the medium. Cells of *S. enterica* were also grown at 37 °C either in LB medium or in no-carbon E (NCE) medium (Horswill & Escalante-Semerena, 1997) supplemented with 0.5% (w/v) sodium propionate.

Isolation and manipulation of DNA. Chromosomal DNA of Tn5-induced mutants of *R. eutropha* HF39 was isolated by the method of Marmur (1961). Plasmid DNA was isolated by the method of Birnboim & Doly (1979). DNA restriction fragments were purified with the Nucleotrap-Kit (Machery-Nagel) as described by the manufacturer. Restriction enzymes, ligases and other DNA-manipulating enzymes were used according to the manufacturers' instructions.

Transfer of DNA. Competent cells of *E. coli* were prepared and transformed by the CaCl₂ procedure as described by Hanahan (1983). The transduction of genomic DNA of *R. eutropha* ligated into the cosmid pHC79 to *E. coli* S17-1 was done after *in vitro* packaging in λ phages as described by Hohn & Murray (1977). The packaging extracts were prepared by the method of Scalenghe *et al.* (1981). Electrocompetent cells of *S. enterica* were prepared and regenerated by the method of Taghavi *et al.* (1994) and transformed under the following conditions: 2400 V, 25 µF and 200 Ω.

Tn5 mutagenesis. Tn5-induced mutants of *R. eutropha* HF39 impaired in growth on levulinic acid were created by the suicide plasmid technique employing pSUP5011 (Simon *et al.*, 1983a), which was transferred from *E. coli* S17-1 to the recipient by conjugation (Friedrich *et al.*, 1981).

Genotypic characterization of the Tn5-insertion mutants of *R. eutropha* HF39. Genomic DNA of the Tn5-insertion mutants was digested with *EcoRI*, and the genomic *EcoRI* fragments were ligated to the cosmid pHC79. After *in vitro* packaging in λ phages the recombinant cosmids were transduced into *E. coli* S17-1. Recombinant *E. coli* clones were selected by their kanamycin resistance conferred by Tn5::*mob*. The hybrid cosmids were isolated, digested with *SalI* and ligated into the plasmid pBR325. The recombinant plasmids were transformed into *E. coli* XL-1 Blue, and clones resistant to kanamycin plus chloramphenicol were selected. The hybrid plasmid of the resulting clones harboured a *SalI* fragment which included part of Tn5 plus genomic DNA adjacent to the Tn5 insertion. These recombinant plasmids were sequenced using the oligonucleotide 5'-GTTAGGAG-GTCACATGG-3', which binds specifically to the IS50L element of Tn5::*mob*.

DNA sequencing. The primer-hopping strategy (Strauss *et al.*, 1986) was applied to determine the DNA sequence of both strands of DNA. Sequencing was done by using the Sequi Therm EXCEL TM II long-read cycle sequencing kit (Epicentre Technologies) and IRD 800-labelled oligonucleotides (MWG-Biotech). Sequencing was performed in a LI-COR 4000L automatic sequencing apparatus (MWG-Biotech).

The nucleotide sequences of the *prp* gene cluster were deposited in the GenBank database under the accession numbers AF325554 and AF331923.

Sequence data analysis. Sequence data were compared with sequences deposited in the GenBank and Prosite databases using the programs BlastSearch 2.0.10 (Altschul *et al.*, 1997) and DBGET (Bairoch *et al.*, 1997).

PCR amplifications. All PCR amplifications of DNA encoded on plasmids or genomic DNA were carried out as described by Sambrook *et al.* (1989). VENT_R-DNA polymerase from New England Biolabs was used in all PCR amplifications, which

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
<i>R. eutropha</i>		
HF39	Sm ^r mutant of the wild-type H16, autotrophic, prototrophic	Srivastava <i>et al.</i> (1982)
HF149	HF39 with Tn5-insertion mutation in <i>rpoN</i>	Hogrefe <i>et al.</i> (1984)
SK7286, SK7290, P2, VG17 and VG3	Levulinate-leaky Tn5-insertion mutants of HF39	This study
SK7287, SK4507, 3-27 and 3-39	Levulinate-negative Tn5-insertion mutants of HF39	This study
<i>E. coli</i>		
XL-1 Blue	<i>recA1 endA1 gyrA96 thi hsdR17</i> ($r_k^- m_k^-$) <i>supE44 relA1 $\lambda^- lac$</i> [<i>F'</i> <i>proAB lacI^qΔM15 Tn10(tet)</i>]	Bullock (1987)
S17-1	<i>recA</i> ; harbours the <i>tra</i> genes of plasmid RP4 in the chromosome; <i>proA thi-1</i>	Simon <i>et al.</i> (1983a)
BHB2688	N205, <i>recA'</i> [λ imm ⁴³⁴ , cIts857, b2, <i>red3</i> , Eam4, Sam7/ λ]	Hohn & Murray (1977)
BHB2690	N205, <i>recA'</i> [λ imm ⁴³⁴ , cIts857, b2, <i>red3</i> , Dam15, Sam7/ λ]	Hohn & Murray (1977)
<i>S. enterica</i>		
JE3907	<i>metE205, ara-9, zai-6386::Tn10d(Tc^r) prpC167</i>	Horswill & Escalante-Semerena (1997)
JE4176	<i>prpC</i> ⁺ in pBAD30; <i>bla</i> ⁺ (Ap ^r)	Horswill & Escalante-Semerena (1997)
Plasmids		
pHC79	Cosmid Tc ^r Ap ^r	Hohn & Collins (1980)
pBR325	Ap ^r Tc ^r Cm ^r	Bolivar (1978)
pBluescript SK ⁻	Ap ^r <i>lacPOZ</i> ; T7 and T3 promoter	Stratagene
pBCSK ⁺	Cm ^r <i>lacPOZ</i> ; T7 and T3 promoter	Stratagene
pSKsymΩKm	ΩKm in pSKSym	Overhage <i>et al.</i> (1999)
pSUP202	Ap ^r Tc ^r Cm ^r	Simon <i>et al.</i> (1983b)

were carried out in an Omnigene HBTR3CM DNA Thermal Cycler (Hybaid).

DNA–DNA hybridization. Southern hybridizations were done by the method of Oelmüller *et al.* (1990) at 68 °C. Colony hybridizations were done by the method of Grunstein & Hogness (1975) at 68 °C.

Cloning of *prpB*, *prpC* and *acnM* and heterologous expression in *E. coli*. Oligonucleotides were constructed to amplify *prpB* (*prpBUP*, 5'-AAATCTAGAGGCTTGGCACACCCCTT G-CAGTATTG-3'; *prpBRP*, 5'-TTTTTGTGCGACGGCCGC-GCGTGAGTCTTGCTTAC-3'), *prpC* (*prpCUP*, 5'-AAA-GAATTCCCGGCCGCTTGACAG-3'; *prpCRP*, 5'-TTT-TTGTCGACCAACTACCCCTTGTC-3') and *acnM* (*acnMUP*, 5'-AAGAGTCTCGCTGACATGGG ACCACTA-CC-3'; *acnMRP*, 5'-AAATCTAGACGGGCCCTTTGTCA-CAGCTTATGC-3') from genomic DNA of *R. eutropha* HF39 (restriction sites are underlined). The 984 bp DNA fragment resulting from *prpBUP* and *prpBRP* was cloned into pBluescript SK⁻, resulting in pSK⁻/*prpB*. *prpCUP* contained an *EcoRI* recognition sequence and *prpCRP* a *SalI* recognition sequence to enable forced cloning of *prpC* downstream of and collinear to the *lacZ* promoter with *EcoRI* plus *SalI* restricted pBluescript SK⁻ DNA, resulting in pSK⁻/MCSII. *acnMUP* contained a *SacI* recognition sequence and *acnMRP* a *XbaI* recognition sequence to enable forced cloning of *acnM* downstream of and collinear to the *lacZ* promoter of *SacI* plus *XbaI* restricted pBluescript SK⁻ DNA; the resulting hybrid plasmid was referred to as pSK⁻/*acnM*.

Cells of *E. coli* harbouring pSK⁻/*prpB*, pSK⁻/MCSII, pSK⁻/*acnM* or pBluescript SK⁻ were grown at 30 °C for 16 h in 50 ml LB medium containing 75 µg ml⁻¹ ampicillin plus 0.5 mM IPTG. The cells were harvested (10 min, 4500 r.p.m. at 4 °C), washed with 100 mM Tris/HCl (pH 8.0), resuspended in 5 ml 100 mM Tris/HCl and disrupted by twofold passage through a French press.

Inactivation of the *prpB* gene of *R. eutropha* HF39 by insertion of the omega element ΩKm. For inactivation of *prpB* by insertion of ΩKm, the 820 bp *EcoRI* fragment (EE0-82) was used, which contained the incomplete *prpB* gene (Fig. 1, A). This fragment was ligated to *EcoRI*-digested vector pSKSym, and *E. coli* XL-1 Blue was transformed with the ligation mixture. Transformants harbouring the hybrid plasmid pSym*prpB* were obtained. Plasmid pSym*prpB* was then digested with *EcoRV* and ligated with ΩKm, which was recovered by *SmaI* digestion of plasmid pSKsymΩKm (Overhage *et al.*, 1999). The hybrid plasmid thus obtained was designated pSym*prpB*ΩKm. The disrupted *prpB* gene was isolated from pSym*prpB*ΩKm by *EcoRI* digestion and ligated with *EcoRI*-digested pSUP202 DNA. *E. coli* S17-1 was transformed with the ligation mixture, and transformants harbouring the hybrid plasmid pSUP*prpB*ΩKm, which conferred resistance to tetracycline and kanamycin, were obtained. Subsequently, pSUP*prpB*ΩKm was transferred to *R. eutropha* HF39 by conjugation, and the transconjugants were selected on NB agar plates containing 160 µg kanamycin ml⁻¹. The transconjugants were tested for tetracycline resistance (encoded by a vector-borne gene) to distinguish between the

integration of the whole hybrid plasmid into the chromosome by a single crossover (heterogenotes) and the exchange of the functional *prpB* gene with the disrupted gene by a double crossover (homogenotes), which resulted in a tetracycline-sensitive phenotype. To confirm the disruption of the *prpB* gene in the tetracycline-sensitive mutant HF39 Δ *prpB* Ω Km, a PCR was performed using the primer *prpBUP* and *prpBRP*, which resulted in a single PCR product with the expected size of 1.9 kbp. The sequence of the PCR product was determined, revealing the sequence of *prpB* Ω Km.

Inactivation of the *prpD* gene of *R. eutropha* HF39 by insertion of the omega element Ω Km. For inactivation, *prpD* was amplified from genomic DNA using oligonucleotides *prpDUP* (5'-AAACTGCAGACGCGTTCTGAGCTGAGCTGAG-3') and *prpDRP* (5'-AAACTGCAGCATGAAGTTGCCCGGGCGATAACTC-3'); the 1511 bp PCR product was digested with *Pst*I, ligated to *Pst*I-linearized vector pBCSK⁺ and *E. coli* XL-1 Blue was transformed with the ligation mixture. Transformants harbouring the hybrid plasmid pBCSK*prpD* were obtained. Plasmid pBCSK*prpD* was then digested with *Stu*I, resulting in a deletion of a 416 bp fragment of *prpD*, and ligated with Ω Km, which was recovered by *Sma*I digestion of plasmid pSKsym Ω Km (Overhage *et al.*, 1999). The hybrid plasmid thus obtained was designated pBCSK*prpD* Ω Km. The disrupted *prpD* gene was isolated from pBCSK*prpD* Ω Km by *Pst*I digestion and ligated with *Pst*I-digested pSUP202 DNA. *E. coli* S17-1 was transformed with the ligation mixture, and transformants harbouring the hybrid plasmid pSUP*prpD* Ω Km, which conferred resistance to tetracycline and kanamycin, were obtained. Subsequently, pSUP*prpD* Ω Km was transferred to *R. eutropha* HF39 by conjugation. The transconjugants were selected on NB containing 160 μ g kanamycin ml⁻¹ and were then tested for tetracycline resistance. PCR was performed with genomic DNA of tetracycline-sensitive and -resistant transconjugants using primers which bind to the *tet* gene (tc-up, 5'-GCTAACGACGTCAGGCACCGTGTATG-3'; tc-down, 5'-CGTTAGCGAGGTGCCGCCGGCTTC-3') of the vector pSUP202. No PCR product was obtained using genomic DNA of tetracycline-sensitive transconjugants, whereas the *tet* gene was amplified from genomic DNA of a tetracycline-resistant transconjugant. To confirm the disruption of the *prpD* gene in the tetracycline-sensitive mutant HF39*prpD* Ω Km, a PCR was performed using the primers *prpDKOup* (5'-GTGACGCGCAATGGGCGGTCAAGAAG-3') and *prpDKOdown* (5'-CGCGGGTCGGCAGCAATCCTGTCTTC-3'), which resulted in a single PCR product with the expected size of 1.85 kbp. The sequence of the PCR product was determined, revealing the sequence of *prpD* Ω Km.

Preparation of crude extracts. Cells from 20 ml cultures were washed in 10 mM Tris/HCl (pH 7.4) and resuspended in 1 ml of this buffer containing 10 μ g DNase I. The cells were disrupted by sonification in a Sonopuls GM 200 (Bandelin, Berlin, Germany) with an amplitude of 16 μ m (1 min ml⁻¹). During ultrasonification the samples were cooled in a NaCl/ice bath. Soluble protein fractions of the crude extracts were obtained by 5 min centrifugation at 13000 r.p.m. and 4 °C.

Cultivation of *R. eutropha* HF39, SK7286 and HF39 Δ *prpB* Ω Km to determine the enzyme activity of 2-methylcitrate synthase and 2-methylisocitrate lyase. The specific enzyme activities of 2-methylcitrate synthase and 2-methylisocitrate lyase were measured in *R. eutropha* HF39, the *prpC* mutant SK7286 and HF39 Δ *prpB* Ω Km to confirm the inactivation of *prpC* or *prpB* in these mutants. Cells of these strains were grown in 50 ml MM containing 1.0% (w/v) sodium gluconate for 30 h at

30 °C. The cells were harvested, washed twice with sterile saline and transferred to fresh MM containing 0.2% (w/v) sodium levulinate, sodium propionate or sodium gluconate. After incubation for 24 h at 30 °C the cells were harvested, and the enzyme activities were measured in the crude extracts.

Determination of the enzyme activity of 2-methylcitrate synthase, 2-methylisocitrate lyase and 2-methyl-*cis*-aconitic acid hydratase. Activity of 2-methylcitrate synthase (EC 4.1.3.31) was measured by the method of Srere (1966). The cuvette ($d = 1$ cm) contained, in a total volume of 1 ml, 2 mM oxaloacetate, 250 μ M propionyl-CoA and 2 mM 5,5'-dithiobis-(2-nitrobenzoate) (DTNB) in 10 mM Tris/HCl (pH 7.4). After addition of the crude extract, the increase of the absorbance at 412 nm ($\epsilon = 13.6$ cm² μ mol⁻¹) was measured with an Ultrospec 2000 photometer (Pharmacia).

Activity of 2-methylisocitrate lyase was assayed in a total volume of 0.5 ml containing 66.7 mM potassium phosphate buffer (pH 6.9), 3.3 mM phenylhydrazine, 2.5 mM cysteine, 5 mM MgCl₂ and cell extract. The reaction was started by addition of 1.25 mM tripotassium methylisocitrate, which was obtained by alkaline cleavage of the methylisocitric acid lactone (Brock *et al.*, 2001; W. Buckel, personal communication). The increase of the absorbance at 324 nm was measured. The molar absorption coefficient of pyruvate phenylhydrazone was 12 mM⁻¹ cm⁻¹ (Luttik *et al.*, 2000).

Activity of the putative 2-methyl-*cis*-aconitic acid hydratase was measured in a quartz cuvette ($d = 1$ cm) in 1 ml 100 mM Tris/HCl buffer (pH 8.0) containing 0.1 mM *cis*-aconitic acid or 1.5 mM tricalcium salt of 2-methylcitric acid plus crude extract. After addition of the substrate, the decrease (*cis*-aconitic acid; $\epsilon = 4.1$ cm² μ mol⁻¹) or the increase (tricalcium salt of 2-methylcitric acid; $\epsilon = 4.5$ cm² μ mol⁻¹) of the absorbance at 240 nm were measured.

One unit of enzyme activity was defined as the conversion of 1 μ mol substrate min⁻¹. The amount of soluble protein was determined by the method of Bradford (1976), using crystalline bovine serum albumin as standard.

Preparation of the tricalcium salt of 2-methylcitric acid. Cells of the Tn5-mutant VG17 of *R. eutropha* HF39 were grown for 24 h at 30 °C in 1 l MM containing 1.0% (w/v) disodium succinate plus 160 μ g kanamycin ml⁻¹. The cells were harvested (20 min, 4000 r.p.m.), washed with sterile saline and transferred to 500 ml fresh MM containing 0.2% (w/v) sodium propionate. After incubation for 24 h at 30 °C, 0.6% (w/v) disodium succinate and 0.4% (w/v) sodium propionate were added. After further incubation for 48 h at 30 °C, the cells were sedimented by centrifugation, and the supernatant was lyophilized. The supernatant was resuspended in 0.1 vol. double-distilled water, and 2-methylcitric acid was extracted by the method of Brock *et al.* (2000). To purify this extract, 2 vols double-distilled water was added, and the solution was neutralized with CaOH₂. Addition of 1 vol. acetone or 2-propanol resulted in the precipitation of a slightly yellowish powder, which could be purified to a white powder by repeating this precipitation step. GC/MS analysis (see below) of the white powder identified this substance as 2-methylcitric acid.

Analysis of the supernatant of resting cells. Cells of the Tn5-insertion mutants VG17, P2 and SK7287, the null-allele mutants HF39 Δ *prpB* Ω Km and HF39*prpD* Ω Km, and *R. eutropha* HF39 were grown in 50 ml MM containing 0.5% (w/v) disodium succinate as carbon source for 24 h at 30 °C. The cells were harvested, washed twice with sterile saline and

transferred to fresh MM containing 0.1% (w/v) sodium propionate. After 24 h incubation at 30 °C 0.1% (w/v) sodium propionate and 0.25% (w/v) disodium succinate were added. Aliquots of 1 ml were withdrawn at different times and centrifuged for 5 min at 13 000 r.p.m. The clear supernatants were lyophilized, subsequently esterified and used for GC/MS analysis.

GC/MS analysis. The white powder and cell-free lyophilized supernatants were subjected to methanolysis in the presence of sulfuric acid (5 h at 100 °C), and the resulting methyl esters of the organic acids were characterized by coupled gas chromatography/mass spectrometry (GC/MS) using an HP 6890 gas chromatograph equipped with a model 5973 mass selective detector (Hewlett Packard).

Electrophoretic methods. Proteins were separated under denaturing conditions in polyacrylamide gels, which were stained with Coomassie brilliant blue R as described by Osborn & Weber (1969).

RESULTS AND DISCUSSION

Phenotypic characterization of levulinate-negative and -leaky mutants

Transposon mutagenesis of *R. eutropha* HF39 was performed to obtain mutants affected in the metabolism of levulinate. Several Tn5-induced null-allele mutants of *R. eutropha* HF39 exhibiting the phenotypes levulinate-negative (18) or levulinate-leaky (7) were isolated which grew like the wild-type with gluconate, octanoate or citrate as sole carbon source. Some of them were studied in detail. On MM agar plates containing levulinate, 4-hydroxyvalerate, valerate or propionate as sole carbon and energy source, the mutants SK4507, 3-29, 3-27 and SK7287 exhibited no growth, whereas the mutants VG17, P2, SK7286, SK7290 and VG3 grew much more slowly than the wild-type.

Molecular characterization of Tn5-induced mutants defective in the catabolism of levulinic acid

The insertion of Tn5 into the genomes of these mutants was confirmed by Southern hybridization using *Eco*RI-digested DNA isolated from the mutants and the central digoxigenin (DIG)-labelled 5.1 kbp *Hind*III fragment of Tn5 as probe.

To map the insertions of Tn5 in seven of these mutants, *Sal*I restriction fragments conferring kanamycin resistance were cloned. Sequencing of these *Sal*I fragments, employing an oligonucleotide hybridizing to the terminal IS50L region of Tn5, revealed strong homologies to structural genes involved in the catabolism of propionate. In the mutants SK7286 and SK7290 Tn5 mapped at an identical position, and the amino acid sequence of the putative translational product revealed 78.0% similarity to the *prpC* gene products of *S. enterica* serovar Typhimurium and *E. coli* (Horswill & Escalante-Semerena, 1997; Textor *et al.*, 1997), which encodes a 2-methylcitrate synthase. The sequence of the Tn5 insertion locus in the mutants VG17 and P2 revealed strong similarities to aconitate hydratase genes (Prodromou *et al.*, 1992) and genes encoding iron-

responsive element binding proteins (Yu *et al.*, 1992). The Tn5-insertion locus in the mutant SK7287 mapped 38 bp downstream of the *Sal*I recognition sequence and showed no similarities to other genes. However, the insertion had probably occurred in proximity to those in VG17 and P2 as indicated by the identical sizes of the Tn5-containing *Eco*RI and *Sal*I fragments. In the mutants VG3, 3-29, 3-27 and SK4507, Tn5 mapped in a gene whose putative translational product showed strong similarities to the *prpR* gene product of *S. enterica* and *E. coli* (Horswill & Escalante-Semerena, 1997; Textor *et al.*, 1997).

Cloning and sequencing of native genomic fragments

A DIG-labelled Tn5::*mob*-harbouring *Eco*RI restriction fragment cloned from mutant VG17 was used to identify the native 3.7 kbp *Eco*RI fragment referred to as VG17EE3.7 in a genomic library in *E. coli* S17-1 prepared from *Eco*RI-digested DNA in the cosmid pH79 (Fig. 1, A). It was subsequently ligated to linearized pBluescriptSK⁻ and transferred to *E. coli* XL-1 Blue. Another gene library was prepared from *Hind*III-digested genomic DNA of strain HF39 and cosmid pH79 in *E. coli* S17-1. Colony hybridization with DIG-labelled EE3.7 DNA identified a clone which harboured a 8.5 kbp *Hind*III fragment (HH8.5) containing a 3.2 kbp *Hind*III-*Eco*RI fragment as part of the native 3.7 kbp *Eco*RI fragment (Fig. 1, A). With DIG-labelled EE10.1 Tn5::*mob*-containing DNA of the mutant SK4507, an 8.4 kbp *Hind*III fragment was identified. When it was digested with *Eco*RI, five fragments, of 2.8, 2.6, 0.82, 0.372 and 0.218 kbp, were obtained. With the DIG-labelled *prpC* gene as a probe a 2.9 kbp *Hind*III restriction fragment (HH2.9) was identified in the hybrid plasmid of the positive *E. coli* clone (Fig. 1, A). The restriction fragments shown in Fig. 1(A) were cloned into the vectors pBluescript SK⁻ or pBCSK⁺ and fully sequenced. Six ORFs were identified, five of which showed similarities to structural genes (Fig. 1, B).

Function of the putative gene product of ORF1

The deduced amino acid sequence of this 1962 bp ORF, which started with an ATG at position 1962 (see Fig. 1, included as supplementary data with the on-line version of this paper at <http://mic.sgmjournals.org>), showed 40–43% identity to the *prpR* gene product of *E. coli* and *S. enterica* serovar Typhimurium and to various other transcriptional activators belonging to the σ^{54} -(*rpoN*) family (Palacios & Escalante-Semerena, 2000). The *prpR* gene product is probably a transcriptional activator of the *prpBCAOD* operon in *R. eutropha* HF39 and belongs to the σ^{54} family of transcriptional activators. A feature of σ^{54} -dependent transcription, in addition to a -24/-12 promoter sequence, is its dependency on an activator protein. Most of these activator proteins bind to conservative sequences with a twofold rotational symmetry, which are designated upstream activator sequences (UAS) and are located 80–120 bp upstream of the σ^{54} -dependent promoter

Fig. 1. The *prp* locus of *R. eutropha* HF39. (A) Native DNA fragments as identified by colony hybridisation or relevant for nucleotide sequence analysis. (B) Organization of *prpR*, *prpB*, *prpC*, *acnM*, ORF5 and *prpD* and locations of transposon and omega element insertions as determined by sequence analysis of DNA fragments isolated from mutants.

(Kustu *et al.*, 1989). At 83 bp upstream of the putative σ^{54} -dependent promoter of the *prpBCAOD* operon in *R. eutropha* HF39 a putative UAS was identified (TGT-N₁₂-ACA), which strongly resembled the UAS of *nifA* (TGT-N₁₀-ACA) of bacteria (Alvarez-Morales *et al.*, 1986) and the putative UAS of *acoD* of *R. eutropha* (TGT-N₁₁-ACA) (Priefert & Steinbüchel, 1992). A second putative UAS was located 102 bp upstream of the putative σ^{54} -dependent promoter (TTGAATTNCAAA-N₈-TTTGNAATTCAA). This imperfect palindromic sequence overlapped with the putative σ^{70} -dependent promoter of the *prpR* gene. The binding of an activator protein to this putative UAS could repress the σ^{70} -dependent transcription of *prpR*. The propionate-negative phenotype of the *rpoN*-negative mutant HF149 of *R. eutropha* (not shown in detail) provided further evidence for σ^{54} -dependent transcription of *prp* genes.

Function of the putative gene product of ORF2

The amino acid sequence deduced from the 909 bp nucleotide sequence of ORF2 exhibited identities of 76.7% and 75.9% and similarities of 87.7% and 86.2% to the *prpB* gene products of *E. coli* (Textor *et al.*, 1997) and *S. enterica* (Horswill & Escalante-Semerena, 1997), respectively. Furthermore, the *prpB* gene product also showed significant identities to carboxyphosphoenolpyruvate phosphonmutases (32–40%) and isocitrate lyases (25–31%) of several organisms. The function of the *prpB* gene product as a 2-methylisocitric acid lyase was suggested for *E. coli* (Textor *et al.*, 1997).

From the amino acid alignment (see Fig. II, included as supplementary data with the on-line version of this paper at <http://mic.sgmjournals.org>) and from the

tentative ribosome-binding site, which preceded the ATG at position 2243, it was concluded that this codon is probably the putative translational start codon of *prpB* of *R. eutropha*. PrpB consisted of 302 amino acids with a calculated molecular mass of 32 314 Da and a pI of 5.15. Thirty-six nucleotides upstream of the 5'-terminal region of *prpB* a consensus σ^{54} promoter sequence was identified as in *S. enterica* serovar Typhimurium LT2 (Palacios & Escalante-Semerena, 2000).

prpB was heterologously expressed in *E. coli* XL-1 Blue (pSK⁻/*prpB*), and a protein of approximately 32 ± 1 kDa was synthesized from the recombinant *E. coli* strain, which corresponded well with the molecular mass calculated for the *prpB* gene product (see Fig. IIIA, included as supplementary data with the on-line version of this paper at <http://mic.sgmjournals.org>).

Inactivation of the *prpB* gene of *R. eutropha* HF39 by insertion of the omega element Ω Km. Since no Tn5 insertion mapped in *prpB*, the phenotype of a mutant with defective *prpB* was unknown. For inactivation of *prpB*, pSUP*prpB* Ω Km was transferred to *R. eutropha* HF39 by conjugation and the null-allele mutants were selected as described in Methods. The homogenote HF39 Δ *prpB* Ω Km could not grow on MM agar plates containing sodium propionate as a sole carbon source.

Determination of 2-methylisocitrate lyase activity. The activity of 2-methylisocitrate lyase was measured in crude extracts of propionate-induced [0.140 U (mg protein)⁻¹] and uninduced cells [0.0024 U (mg protein)⁻¹] of *R. eutropha* HF39 and propionate-induced cells of HF39 Δ *prpB* Ω Km [no activity measurable]. The results revealed that the formation of 2-methylisocitrate lyase is induced by propionic acid in *R. eutropha* HF39.

Fig. 2. (a, b) GC/MS analysis of the supernatants of the Tn5 mutant VG17 (a) and the knock-out mutant HF39 Δ *prpB* Ω Km (b). (c) Mass spectrum of the putative 2-methylisocitric acid trimethyl ester.

Furthermore, 2-methylisocitrate lyase activity of 0.191 U (mg protein)⁻¹ was determined in the recombinant *E. coli* (pSK⁻/*prpB*) in comparison to only 0.0036 U (mg protein)⁻¹ in an *E. coli* strain harbouring only pBluescript SK⁻.

Physiological investigation of the *prpB* null-allele mutant.

To get a hint of the physiological function of the *prpB* translational product in the methylcitric acid cycle of *R. eutropha*, three-stage growth experiments were carried out as described in Methods with the null-allele mutant HF39 Δ *prpB* Ω Km and strain *R. eutropha* HF39 as control. In the mutant HF39 Δ *prpB* Ω Km six components accumulated in the supernatant (Fig. 2b). The mass spectrum of the compound with a retention time of 35.35 min in the gas chromatogram revealed an identity of 92.8% to the trimethyl ester of 2-methylcitric acid (NIST database). Comparison of the peak at 36.00 min with the mass spectrum of the trimethylester of isocitric acid (NIST database) revealed that nearly all key fragments showed a 14–15 higher *m/z* (Fig. 2c), which corresponds to the mass of the additional methyl group in 2-methylisocitric

acid (not contained in the database). This suggests that this component of the supernatant is 2-methylisocitric acid. The component with a retention time of 39.91 min showed an identical mass spectrum as the component with a retention time of 36.00 min, except that two fragments with *m/z* 146 and *m/z* 189 were missing. Esterification of the trisodium salt of isocitric acid and GC/MS analysis revealed a retention time for the methyl ester of this substance of 40.85 min and the key fragments had an *m/z* which was 14–15 lower than the *m/z* of the peaks in the 39.91 min peak mass spectrum. The lactone of 2-methylisocitric acid and isocitric acid might be formed as a side product of esterification, as described for esterification of these substances in the supernatant of *Yarrowia lipolytica* R-2 (Tabuchi & Serizawa, 1975), whereas the dimethyl ester of the lactone of 2-methylisocitric acid gave a single sharp peak with a later retention time than the trimethyl ester of 2-methylcitric acid (Tabuchi & Serizawa, 1975). These properties of the dimethyl ester of the methylisocitric acid lactone correspond well with the properties of the dimethyl ester of the putative 2-methylisocitric acid lactone in the supernatant of the mutant HF39 Δ *prpB* Ω Km. Furthermore, 2-methylisocitric acid and 2-methylisocitrate lactone, which was a gift from W. Buckel (Phillips-Universität Marburg, Germany), were used as GC/MS standards and they revealed identical retention times and mass spectra as the substances with retention times of 36.00 and 39.91 min in the supernatant of HF39 Δ *prpB* Ω Km. In the supernatant of *Y. lipolytica* R-2, which produced 2-methylisocitric acid from odd-carbon n-alkanes, citric acid, 2-methylcitric acid and 2-methyl-*cis*-aconitic acid were additionally identified (Tabuchi & Serizawa, 1975). The disruption of *prpB* caused the accumulation of 2-methylisocitric acid and probably as a consequence of the equilibrium 2-methylcitric acid was accumulated. This result provides clear evidence that the *prpB* gene product is involved in the cleavage of 2-methylisocitric acid into succinate and pyruvate in the methylcitric acid cycle of *R. eutropha* HF39 (Fig. 3) as shown for the translational product of *ICL2* in *Sacch. cerevisiae* (Luttik *et al.*, 2000).

Function of the putative gene product of ORF3

The deduced amino acid sequence of ORF3 (1155 bp), which started with an ATG at position 3249 (see Fig. 1, included as supplementary data with the online version of this paper at <http://mic.sgmjournals.org>), showed 78% similarity to the *prpC* gene products of *E. coli* and *S. enterica* (see Fig. IV, included as supplementary data with the online version of this paper at <http://mic.sgmjournals.org>) encoding the methylcitrate synthase.

Determination of 2-methylcitrate synthase activity and expression of *prpC* in *E. coli*. In crude extracts, *R. eutropha* HF39 exhibited significantly higher specific activity of 2-methylcitrate synthase [0.354 U (mg protein)⁻¹] than SK7286 [0.01 U (mg protein)⁻¹]. In addition, the specific

Fig. 3. Methylcitric acid cycle in *R. eutropha* HF39. The bold cross-bars indicate metabolic block caused by insertion of Tn5 or Ω Km.

2-methylcitrate synthase activity of HF39 was approximately twofold higher in cells cultivated on propionate [$0.354 \text{ U (mg protein)}^{-1}$] than in those cultivated on levulinate [$0.145 \text{ U (mg protein)}^{-1}$].

The Tn5-containing *SalI* fragment of mutant SK7286 was subcloned in pBluescript SK⁻ and sequenced. Analysis of the nucleotide sequence made it possible to design oligonucleotides for the amplification of *prpC* from genomic DNA of *R. eutropha* by PCR. *prpC* was cloned into pBluescript SK⁻, resulting in pSK⁻/MCSII. The recombinant *E. coli* strain harbouring pSK⁻/MCSII synthesized a protein of approximately $42 \pm 1 \text{ kDa}$, which corresponded well with the calculated molecular mass of 42720 Da for the *prpC* gene product. Crude extracts of *E. coli* (pSK⁻/MCSII) exhibited a 2-methylcitrate synthase specific activity of $2.86 \text{ U (mg protein)}^{-1}$, if propionyl-CoA was used as substrate, whereas with acetyl-CoA the specific activity was only approximately 10% of this value. Crude extracts of an *E. coli* strain harbouring only pBluescript SK⁻ exhibited no 2-methylcitrate synthase activity using propionyl-CoA as substrate and $0.009 \text{ U (mg protein)}^{-1}$ with acetyl-CoA as substrate.

Phenotypic complementation of the Tn10-induced null-allele *prpC* mutant JE3907 of *S. enterica*. When pSK⁻/MCSII was

transferred to the Tn10-induced null-allele *prpC* mutant JE3907 of *S. enterica* by electroporation, phenotypic complementation of this mutant occurred, as revealed by growth of the recombinant strain in no-carbon E medium containing 0.5% (w/v) sodium propionate. However, the growth of the complemented mutant exhibited a lag phase of about 180 h, whereas the lag phase was only 50 h in a recombinant mutant strain JE4176, harbouring *prpC* of *S. enterica* in pBAD30. In contrast, JE3907 harbouring only the vector did not grow at all, even after a prolonged incubation period.

Function of the putative gene product of ORF4

Downstream and at a distance of 118 bp from ORF3, ORF4 started with an ATG at position 4525, and it was preceded by a putative ribosome-binding site (see Fig. 1, included as supplementary data with the online version of this paper at <http://mic.sgmjournals.org>). The putative translational product of ORF4 has a calculated size of 94726 Da and a pI of 5.36. The comparison of the deduced amino acid sequence of ORF4 with the primary structures of other proteins exhibited the highest similarity, of 85% and 80.3%, to the aconitate hydratases of *Pseudomonas putida* KT2440 and *Neisseria meningitidis* serogroup B (see Fig. V, included as supplementary data

Fig. 4. Comparison of the *prp* loci of several organisms: *R. eutropha* HF39 (B1); *R. eutropha* CH34 (B2); *N. meningitidis* (B3); *P. putida* KT2440 (B4); *P. aeruginosa* (B5); *V. cholerae* (B6) and *S. enterica* (B8).

with the online version of this paper at <http://mic.sgmjournals.org>), whose genes are located downstream of the *prpC* gene in a cluster (Fig. 4). In contrast, the identity of the gene product of ORF4 to Aco1 of *E. coli* (Prodromou *et al.*, 1992) and to AcoN of *L. pneumophila* (Mengaud & Horwitz, 1993), which catalyse the isomerization step in the citric acid cycle, was only 45%. A dendrogram of the amino acid sequences of several aconitate hydratases revealed that the putative *acnM* translational products form a cluster, which separates them from aconitases acting in the citric acid cycle (Fig. 5). The amino acid sequences of aconitate hydratases include three highly conserved cysteine residues, which represent the ligands for the 4Fe–4S cluster. These three cysteine residues also occurred in the *acnM* gene product of *R. eutropha* (C477, C480 and C491). These similarities of ORF4 to aconitate hydratases gave a hint that the encoded enzyme catalyses a hydration/dehydration reaction (2-methylcitric acid → 2-methyl-*cis*-aconitic acid → 2-methylisocitric acid) as part of the methylcitric acid cycle in *R. eutropha* HF39; it was therefore referred to as *acnM*.

A protein of approximately 94 ± 1 kDa was synthesized by the recombinant *E. coli* strain harbouring pSK[−]/*acnM*, after induction with 1 mM IPTG, which

corresponded well with the molecular mass of 94726 Da calculated for the *acnM* translational product (see Fig. IIIB, included as supplementary data with the online version of this paper at <http://mic.sgmjournals.org>).

The crude extract of *E. coli*(pSK[−]/*acnM*) was used immediately after preparation, because Fe–S clusters of aconitases are known to be sensitive to oxygen (Kennedy *et al.*, 1983). The enzyme activity in the crude extracts was determined as described in Methods using the tricalcium salt of 2-methylcitric acid or *cis*-aconitic acid (Sigma) as substrate. With tricalcium 2-methylcitrate no enzyme activity was measurable in crude extracts of either strain. A reason for this could be that the tricalcium 2-methylcitrate preparation contained an inhibitor for the *acnM* gene product. As 2-methyl-*cis*-aconitic acid was not available, the analogous substrate *cis*-aconitic acid was used at a concentration of 0.1 mM. The specific enzyme activity in crude extracts of *E. coli* carrying pSK[−]/*acnM* was 21.5-fold higher [2.84 U (mg protein)^{−1}] than that in crude extracts of *E. coli* harbouring pBluescript SK[−] [0.132 U (mg protein)^{−1}]. This result indicates that the *acnM* gene product might catalyse the hydration of 2-methyl-*cis*-aconitic acid to 2-methylisocitric acid, and it was therefore designated 2-methyl-*cis*-aconitic acid hydratase.

Fig. 5. Dendrogram of aconitases of several organisms. *Pseudomonas aeruginosa* PAO1 (AcnM, AAG04183; AcnA, AAG04951), *P. putida* KT2440 (contig10768: www.tigr.org/cgi-bin/BlastSearch/BLAST.cgi?organisms=p_putida_KT2440), *R. eutropha* HF39 (AF325554), *Ralstonia metallidurans* CH34 (contig371: www.jgi.doe.gov/tempweb/JGI_microbial/html/index.html), *Neisseria meningitidis* (CAC08952), *Vibrio cholerae* (Q9KSC0), *Aeropyrum pernix* (Q9YBI0), *Bacillus subtilis* (BCAODEGR_11), *Streptomyces coelicolor* (Q9RNH9), *Streptomyces viridochromogenes* (Q9RIL1), *Mycobacterium tuberculosis* (053166), *Xylella fastidiosa* (Q9PGK8), *Xanthomonas campestris* (050579) and *E. coli* (ECACN_2).

Function of the putative gene product of ORF5 and identification of the Tn5-insertion locus in the mutant SK7287

The Tn5-insertion locus of the mutant SK7287 has been identified in the 3'-terminal region of the 1191 bp ORF5 (Fig. 1, B; see also Fig. I, included as supplementary data with the online version of this paper at <http://mic.sgmjournals.org>), which started with an ATG at position 7195. The deduced amino acid sequence (396 aa) exhibited the highest similarity, of 71.5%, to a conserved hypothetical 40.9 kDa protein in the *prp* locus of *N. meningitidis* serogroup B (see Fig. VI, included as supplementary data with the online version of this paper at <http://mic.sgmjournals.org>). Furthermore, it showed 33% identity to the hypothetical 39.5 kDa *yraM* gene product of *Bacillus subtilis* (Parro *et al.*, 1997) and a similarity of 36% to a hypothetical 37.1 kDa protein in the *modC-bioA* intergenic region in the genome of *E. coli* (Blattner *et al.*, 1997). The amino acid alignment of the hypothetical 39.5 kDa protein of *B. subtilis* revealed weak similarities (24%) to the *pduG* gene product of *S. enterica* (Bobik *et al.*, 1997), which catalyses the reactivation of a diol dehydratase.

Based on computer analysis with the program PSORT (Klein *et al.*, 1985) the product of ORF5 may be

associated with the membrane, but there are no data available to support this suggestion.

Physiological investigations of null-allele mutants of *acnM* and ORF5. To analyse the physiological functions of the *acnM* and ORF5 translational products in the methylcitric acid cycle of *R. eutropha* HF39, three-stage growth experiments were carried out as described in Methods, with the Tn5 mutants VG17, P2 and SK7287 and *R. eutropha* HF39 as control. All the Tn5 mutants converted succinate into fumarate, malate and 2-methylcitrate. After a period of 48 h, fumarate and malate had completely disappeared in the supernatants of the mutants VG17, P2 and SK7287 and were completely converted into 2-methylcitrate (Fig. 2a). These results showed clearly that both the *acnM* and the ORF5 mutant are impaired in the conversion of 2-methylcitric acid into 2-methylisocitric acid. Polar effects in the mutants VG17, P2 and SK7287 can be excluded, because a defect in the gene located downstream (*prpD*) caused no accumulation of 2-methylcitric acid. As ORF5 is always localized together with *acnM* (Fig. 4), it is conceivable that both gene products are required for the conversion of 2-methylcitric acid into 2-methylisocitric acid.

Function of the putative gene product of ORF6

ORF6 (1455 bp), which started with the ATG at position 8412 (see Fig. I, included as supplementary data with the online version of this paper at <http://mic.sgmjournals.org>), encoded a protein with a theoretical molecular mass of 53 001 Da and a pI of 6.64. The deduced amino acid sequence showed 78.9% and 80.2% similarity to the *prpD* gene products of *E. coli* (Textor *et al.*, 1997) and *S. enterica* (Horswill & Escalante-Semerena, 1997), respectively (see Fig. VII, included as supplementary data with the online version of this paper at <http://mic.sgmjournals.org>), and 60.4% similarity to the *ypo2* gene product of *Sacch. cerevisiae*, a hypothetical 57.7 kDa membrane protein in the *cit3-hal1* intergenic region (Q12428).

Inactivation of the *prpD* gene of *R. eutropha* HF39 by insertion of the omega element Ω Km. Since no Tn5 insertion mapped in ORF6, the phenotype of a mutant with defective ORF6 was unknown. For inactivation of *prpD*, pSUP*prpD* Ω Km was transferred to *R. eutropha* HF39 by conjugation and the null-allele mutants were selected as described in Methods. The homogenote HF39*prpD* Ω Km was not impaired in growth on MM agar plates containing sodium propionate, sodium valerate or sodium levulinate and kanamycin.

The function of *prpD* in *S. enterica* as 2-methylcitric acid dehydratase was proposed by Horswill & Escalante-Semerena (1999b); however, analysis of the *prpD* null alleles showed that a mutation in *prpD* did not block the ability of *R. eutropha* to use propionate as sole carbon source. This ORF is obviously not required for a functional methylcitric acid cycle, or the function of the *prpD* gene product can be taken over by another

enzyme or a non-enzymic hydration of the methylaconitate occurs.

Physiological investigation of the *prpD* null-allele mutant. To get a hint of the physiological functions of the *prpD* translational products, three-stage growth experiments were carried out as described in Methods with HF39*prpD*ΔKm and *R. eutropha* HF39 as control. In the supernatant of HF39*prpD*ΔKm no 2-methylcitric acid or any other organic acid was detectable after a period of 48 h.

Organization of the *prp* locus in Gram-negative bacteria

Analysis of data from genome sequencing projects of various organisms showed that genes homologous to the *prp* genes of *R. eutropha* HF39 are widespread in Gram-negative bacteria. A comparison of these putative *prp* genes revealed two distinct classes of the organization of the *prp* clusters (Fig. 4). In the enterobacteria *E. coli* and *S. enterica* (Blattner *et al.*, 1997; Horswill & Escalante-Semerena, 1997) these loci consist of the structural genes *prpR*, *prpB*, *prpC*, *prpD* and *prpE*, in which *prpBCDE* are organized in an operon (Fig. 4). In the *prp* loci of *R. eutropha* HF39, *Pseudomonas putida* KT2440, *P. aeruginosa*, *N. meningitidis* serogroup B and *Burkholderia sacchari* IPT101^T (data not shown) *prpE* is missing and in *Ralstonia metallidurans* CH34, *N. meningitidis* serogroup B, *Vibrio cholerae* and *B. sacchari* IPT101^T (data not shown) *prpD* is missing (Fig. 4). However, the *prp* loci of these bacteria additionally include *acnM* and ORF5. The *prpE* gene product is not required for the functionality of the methylcitric acid cycle during growth of *R. eutropha* on levulinic acid, as the cycle is inducible during the degradation of levulinic acid. The levulinic acid degradation pathway is not known in detail yet. Furthermore, the function of PrpE can be taken over by the product of the *acoE* gene of *R. eutropha* (Priefert & Steinbüchel, 1992). In *P. putida* KT2440 a second putative aconitate hydratase gene is divergently transcribed from the *prp* locus. A putative regulator gene, which showed identities to regulators of the *gntR* family, is localized collinear to the other genes of the *prp* cluster in the pseudomonads. The *prp* locus of the enterobacterium *V. cholerae* is particularly interesting because it represents a mixture of both classes of *prp* loci: a regulator gene of the *Pseudomonas* type, *acnM*, ORF5 and *prpE* occur in the cluster, but *prpD* is missing.

ACKNOWLEDGEMENTS

This study was supported by a grant provided by the European Commission (FAIR/CT96-1780). We thank Dr Escalante-Semerena for the providing *S. enterica* strains JE 3907 and JE 4176. We are indebted to Dr H. Luftmann (Institut für Organische Chemie, WWU Münster) for helpful discussion of the GC/MS data. We thank W. Buckel and D. Darley (Phillips-Universität Marburg, Germany) for the gift of methylisocitrate lactone.

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Received 22 January 2001; revised 29 March 2001; accepted 23 April 2001.