The glyoxylate bypass of *Ralstonia eutropha*

Zheng-Xiang Wang, Christian O. Brämer, Alexander Steinbüchel

Institut für Molekulare Mikrobiologie und Biotechnologie, Westfälische Wilhelms-Universität Münster, Corrensstraße 3, 48149 Münster, Germany

Research Centre of Industrial Microbiology, School of Biotechnology, Southern Yangtze University, 214036 Wuxi, PR China

Received 20 July 2003; received in revised form 16 September 2003; accepted 17 September 2003

First published online 3 October 2003

**Abstract**

The glyoxylate bypass genes aceA1 (isocitrate lyase 1, ICL1), aceA2 (isocitrate lyase 2, ICL2) and aceB1 (malate synthase, MS1) of *Ralstonia eutropha* HF39 were cloned, sequenced and functionally expressed in *Escherichia coli*. Interposon-mutants of all three genes (\(\Delta aceA1\), \(\Delta aceA2\) and \(\Delta aceB1\)) were constructed, and the phenotypes of the respective mutants were investigated. Whereas *R. eutropha* HF39\(\Delta aceA1\) retained only 19% of ICL activity and failed to grow on acetate, *R. eutropha* HF39\(\Delta aceA2\) retained 84% of acetate-inducible ICL activity, and growth on acetate was not retarded. These data suggested that ICL1 is in contrast to ICL2 induced by acetate and specific for the glyoxylate cycle. *R. eutropha* HF39\(\Delta aceB1\) retained on acetate as well as on gluconate about 41–42% of MS activity and exhibited retarded growth on acetate, indicating the presence of a second hitherto not identified MS in *R. eutropha* HF39. Whereas in *R. eutropha* HF39\(\Delta aceA1\) and *R. eutropha* HF39\(\Delta aceA2\) the yields of poly(3-hydroxybutyric acid), using gluconate as carbon source, were significantly reduced, *R. eutropha* HF39\(\Delta aceB1\) accumulated the same amount of this polyester from gluconate as well as from acetate as *R. eutropha* HF39.

**Keywords:** Glyoxylate bypass; Isocitrate lyase; Malate synthase; Polyhydroxyalkanoate; *Ralstonia eutropha*

1. **Introduction**

Most microorganisms utilizing acetate or other fatty acids as sole carbon source employ the glyoxylate cycle for biosynthesis of precursors of cell compounds. The glyoxylate cycle bypasses the decarboxylation steps of the tricarboxylic acid cycle (TCA), thereby preventing complete oxidation of acetyl-CoA to carbon dioxide. By generating oxaloacetate as acceptor for acetyl-CoA, it permits net incorporation of carbon into cellular metabolic fluxes [1]. Five enzymes are involved in this cycle, three of them are the TCA cycle enzymes citrate synthase (4.1.3.7), aconitase (4.2.1.3) and malate dehydrogenase (1.1.1.37); the other two enzymes, isocitrate lyase (ICL, EC 4.1.3.1) and malate synthase (MS, EC 4.1.3.2) are unique to the glyoxylate cycle. ICL cleaves isocitrate to succinate and glyoxylate, and MS condenses glyoxylate with acetyl-CoA to yield malate. With each turn of the cycle 2 mol of acetyl-CoA is introduced, resulting in the synthesis of 1 mol of the four-carbon compound oxaloacetate [1]. The availability of acetyl-CoA and NADPH is in *Ralstonia eutropha* most important for biosynthesis of poly(3-hydroxybutyric acid) (poly(3HB)) and other polyhydroxyalkanoates (PHAs), and their intracellular concentrations significantly affect the amount and also the composition of PHAs [2]. Acetyl-CoA and NADPH are mainly generated by the Entner–Doudoroff pathway, the pentose phosphate cycle, the pyruvate dehydrogenase complex and the TCA cycle. On carbon sources metabolized primarily to acetyl-CoA, the glyoxylate bypass plays an important role for supply of substrates for PHA synthesis. In this study, we describe the cloning, disruption and functional analysis of genes aceA1, aceA2 and aceB1 encoding ICL isoenzyme 1 (ICL1), ICL isoenzyme 2 (ICL2) and malate synthase 1 (MS1) of *R. eutropha* HF39.

2. **Materials and methods**

2.1. **Bacteria, plasmids and cultivation conditions**

All strains and plasmids investigated are listed in Table 1. *R. eutropha* HF39 and its derivates were grown at

---

* Corresponding author. Tel.: +49 (251) 8339821; Fax: +49 (251) 8383888; E-mail address: steinbu@uni-muenster.de (A. Steinbüchel).
30°C in nutrient broth (NB; 0.8%, v/v) or in mineral salts medium (MM) [3] supplemented with filter-sterilized carbon sources plus 500 μg ml⁻¹ streptomycin. Escherichia coli was cultivated at 37°C in Luria–Bertani (LB) medium [4]. When necessary, ampicillin (75 μg ml⁻¹), kanamycin (50 μg ml⁻¹), chloramphenicol (34 μg ml⁻¹) or tetracycline (25 μg ml⁻¹) was added to the medium.

2.2. Isolation and manipulation of nucleic acids

DNA manipulations and isolation of plasmid DNA were according to [4] and enzymes were used according to the instructions of the manufacturers. For preparation of cosmid DNA a NucleoBond BAC Maxi kit (Clontech Laboratories, USA) was used. R. eutropha HF39 genomic DNA was isolated according to [5] with the following modifications: cells from a 100-ml culture were harvested by centrifugation and incubated at 42°C in the presence of sodium dodecyl sulfate (1%, w/v), RNase A (25 μg ml⁻¹) until the solution became opaque. The supernatant was collected and dialyzed against distilled water. DNA fragments were isolated from agarose gels using a Nucleotrap kit (Macherey-Nagel, Düren, Germany).

2.3. Transfer of DNA

Competent cells of E. coli were prepared and transformed by the CaCl₂ procedure [4]. For transduction of R. eutropha HF39 genomic DNA to E. coli S17-1, DNA was ligated into cosmid pHC79 and packaged by an in vitro packaging kit (Stratagene, USA). DNA transfer into R. eutropha HF39 was achieved by conjugation [6].

2.4. Polymerase chain reaction (PCR) amplifications

PCR amplifications of DNA were carried out as described in a manual [4] using PLATINUM® PfX-DNA polymerase (Gibco BRL Life Technologies, Karlsruhe, Germany) and the primers shown in Table 2.

2.5. In situ DNA hybridization

DNA probes were obtained from genomic DNA of R. eutropha HF39 by PCR based on highly conserved regions from related nucleotide sequences of R. metallidurans CH34 after alignment of the sequences of aceA1, aceA2 or aceB1 from Ralstonia metallidurans CH34, Ralstonia solanacearum, Mycobacterium tuberculosis and Pseudomonas aeruginosa. Oligonucleotides aceA’1-1 and aceA’2-1, aceA’2-2 or aceB’1-1 and aceB’1-2 were used to amplify aceA1, aceA2 or aceB1, respectively. The 740-bp (aceA1) or the 1280-bp (aceA2) and the 640-bp (aceB1) PCR products were purified and inserted into pGEM-T-Easy (Promega, USA) to yield pGEMaceA1’ and pGEMaceB1’, or into pBlueScript SK− to yield pSKaceA2’. Colony hybridization was done at 68°C using digoxigenin (DIG)-labeled aceA1, aceA2 or aceB1 as probes [7] and alkaline phosphatase anti-DIG antibodies plus NBT/BCIP (Roche Diagnosis, Germany).

2.6. DNA sequence analysis

The primer-hopping strategy [8] was applied to determine nucleotide sequences using the Sequi Therm EXCEL TM II long-read cycle sequencing kit (Epicentre Tech-
nologies, WI, USA), IRD800-labeled oligonucleotides (MWG-Biotech, Ebersberg, Germany) and a LI-COR 4000L automatic sequencing apparatus (MWG-Biotech, Ebersberg, Germany). Sequence data analysis and homology searches were performed using the FASTA program and the BLAST network service (National Centre for Biotechnology Information, France). Nucleotide sequences of aceA1, aceA2 and aceB1 were deposited in the GenBank database under accession nos. AJ421510, AF499030 and AJ41511.

2.7. Deletion mutants of aceA1, aceA2 or aceB1 of R. eutropha HF39

Deletion mutants were screened based on replacement of the central regions of the structural genes by a kanamycin-resistance gene (ΩKm) [6]. For deletion of aceA1, oligonucleotides aceA1-5up and 3'aceA1-down were used to remove part of aceA1 by inverse PCR employing pSKaceA1 as template. The PCR product was ligated with ΩKm, resulting in pSKaceA1'::ΩKm. With aceA1(EcoRI) and aceA2(EcoRI) aceA1'::ΩKm was amplified by PCR using pSKaceA1'::ΩKm as template. The PCR product was ligated into EcoRI-restricted pSUP202 to retrieve pSUPCm::aceA1'::ΩKm. For deletion of aceA2, A2up and A2dd were used to remove part of aceA2 by inverse PCR using pSKaceA2 as template. The PCR product was ligated with ΩKm yielding pSKaceA2'::ΩKm. The aceA2'::ΩKm fragment was recovered by digestion of pSKaceA2'::ΩKm with EcoRI and ligated into the EcoRI site of pSUP202 to yield pSUPCm::aceA2'::ΩKm. For deletion of aceB1, aceB-up and aceB-3inv were used to move part of aceB1 by inverse PCR using pSKaceB1 as template. The PCR product was purified and ligated with ΩKm to yield pSKaceB1'::ΩKm. The XhoI-digested aceB1'::ΩKm cassette was ligated into the SalI site of pSUP202 resulting in pSUPCm::aceB1'::ΩKm. Subsequently, pSUPCm::aceB1'::ΩKm or pSUPCm::aceB1'::ΩKm were transferred from E. coli S17-1 to R. eutropha HF39. Transconjugants were selected on NB plates containing 500 µg streptomycin and 160 µg kanamycin per ml. Exchanges of functional aceA1, aceA2 or aceB1 with deleted genes by double crossover were selected and confirmed by DNA sequencing.

2.8. Expression of the R. eutropha HF39 aceA1, aceA2 and aceB1 in E. coli

The complete aceA1, aceA2 or aceB1 genes and adjacent regions were amplified by PCR. Primers aceA1(F) and aceA1(R) containing BamHI sites were used to amplify aceA1 with a 640-bp upstream and a 480-bp downstream sequence. Primers aceA2(F) and aceA2(R) containing EcoRI recognition sites were used to amplify aceA2 with a 320-bp upstream and a 100-bp downstream sequence. Primers aceB1(F) and aceB1(R) containing XhoI restriction sites were used to amplify aceB1 with a 240-bp upstream and a 300-bp downstream sequence. PCR products were digested with BamHI, EcoRI or XhoI and inserted into correspondingly linearized pBlueScript SK- to yield pSKaceA1, pSKaceA2 or pSKaceB1. pSKaceA1 and pSKaceA2 were transformed into the ICL mutant CGSC 5233 of E. coli (aceA7Δ), and pSKaceB1 into the MS mutant CGSC 5234 of E. coli (aceB6Δ).
2.9. Preparation of cell extracts and assay of enzymes

About 0.2 g of wet cells were washed with ice-cold sodium chloride solution (0.85%, w/v) and resuspended in 2 ml of 50 mM morpholinepropane-sulfonic acid (MOPS, pH 7.5) buffer containing 10% (v/v) glycerol, 1 mM EDTA, 1 mM benzamidine and 2 mM dithiothreitol. After passage through a French Press Cell (Aminco, USA) and incubation with 50 μl RNase (10 mg ml⁻¹) and 50 μl DNase (10 mg ml⁻¹) at room temperature, the solution was centrifuged at 27,000×g and 4°C for 30 min, and the supernatant was desalted by passing through a PD-10 column (Amersham Pharmacia Biotech AB, USA).

ICL activity was measured in 50 mM MOPS buffer (pH 7.5) containing 5 mM MgCl₂ and 4 mM phenylhydrazine hydrochloride at 25°C. The reaction was started by addition of 2 mM trisodium D,L-isocitrate, and the formation of 2 mM trisodiumD,L-isocitrate, and the formation of the phenylhydrazone derivative of glyoxylate was monitored at 324 nm (ε = 14.63 cm³ mol⁻¹). MS activity was determined by monitoring the release of CoA during the enzymatic reaction at 25°C using 5,5-dithio-2-nitrobenzoic acid) at 412 nm (ε = 13.6 cm³ mol⁻¹) in 50 mM HEPES buffer (pH 8.0) containing 2 mM EDTA, 100 mM KCl, 0.1 mM 5,5'-dithiobis(2-nitrobenzoic acid), 0.2 mM glyoxylic acid and 0.2 mM acetyl-CoA. One unit of enzyme activity was defined as the conversion of 1 μmol of substrate per minute Protein concentrations were determined by the method of Bradford [9] with crystalline bovine serum albumin fraction V (Serva Feinbiochemica, Germany) as reference.

2.10. Isolation and analysis of PHA

For quantitative determination of PHA, 8–10 mg lyophilized cells were subjected to methanolysis in the presence of 15% (v/v) sulfuric acid at 100°C for 5 h, and the resulting hydroxycylyl methylesters were analyzed by gas chromatography as described previously [10].

2.11. Overexpression of aceA1 in R. eutropha VG12

The aceA1 gene with a 640-bp upstream and a 480-bp downstream sequence was isolated from pSKaceA1 and ligated into pBBR1MCS, resulting in pBBRaceA1, which was then transferred into the Tn5-induced mdh mutant VG 12 of R. eutropha HF39 by conjugation [11].

3. Results

3.1. Identification and characterization of the R. eutropha HF39 ICL genes aceA1 and aceA2 and the MS gene aceB1

To clone the R. eutropha HF39 glyoxylate cycle genes, the PCR products aceA1740, aceA21280 and aceB1640 were obtained from R. eutropha HF39 and used as probes to identify the complete genes in cosmid-mediated genomic libraries of R. eutropha HF39 by colony hybridization. Clones harboring a 32-kb (pHCaceA1), a 35-kb (pHCaceA2) or a 30-kb DNA insert (pHCaceB1), which hybridized with aceA1740, aceA21280 or aceB1640, respectively, were identified. The complete sequences of aceA1, aceA2 and aceB1 were revealed using hybrid cosmids pHCaceA1, pHCaceA2 or pHCaceB1 as templates.

3.2. Characterization of the R. eutropha HF39 aceA1 gene

A 3545-bp nucleotide sequence of pHCaceA1 was analyzed, and the putative aceA1 gene encoding ICL1 of R. eutropha HF39, which is preceded by a tentative ribosome binding site, was identified. AceA1 encodes a protein consisting of 431 amino acid residues with a calculated molecular mass of 47006 Da. The deduced amino acid sequence showed 89.3, 84.6 or 22.8 mol% identity to ICL1 (Icl1p) of R. metallidurans CH34, Icl1p of R. solanacearum and ICL2 (Icl2p) of R. metallidurans CH34, respectively. Analysis of the primary amino acid sequence of ICL1 revealed an ICL signature pattern composed of the amino acid residues K(185), K(186), C(187), G(188), H(189) and M(190) (Fig. 1A) with cysteine187 as putative active site residue [12]. This highly conserved signature pattern is also found in 2-methylisocitric acid lyases (PrpB) which cleaves 2-methylisocitric acid into succinate and pyruvate as the final step of the methylocitric acid cycle (Fig. 1A) [6].

A second open reading frame (ORF1) encoding a protein composed of 292 amino acids with a calculated molecular mass of 32580 Da and exhibiting 49 mol% identity to a probable short-chain dehydrogenase PA3324 of P. aeruginosa (http://www.ncbi.nlm.nih.gov) was identified 301 bp upstream of aceA1. No further ORF could be identified in the 849-bp downstream region of aceA1. The arrangement of aceA1 and ORF1 in the genome was identical to that of R. metallidurans CH34 aceA1; however, different genes were located in the aceA1 downstream region.

3.3. Characterization of the R. eutropha HF39 aceA2 gene

A 2521-bp nucleotide sequence of pHCaceA2 revealed the putative aceA2 gene encoding ICL2 of R. eutropha HF39, which is preceded by a tentative ribosome binding site. The translational product of aceA2 comprised 526 amino acid residues with a calculated molecular mass of 57890 Da. The deduced amino acid sequence was 94.3, 80.0, 22.8 or 23.3 mol% identical to those of R. solanacearum, R. metallidurans CH34, Icl1p, respectively. Analysis of the primary amino acid sequence of R. eutropha HF39 ICL2 exhibited similarities to a putative signature pattern for ICLs composed of the amino acid residues...
A. Isocitrate lyases and methylisocitrate lyases

(1) R. eutropha HF39 ICL 155 GYUJBAUGLSMNIMILAGAVGVEF3Q4LA5YVYKG1MGNVXK
(2) R. eutropha HF39 ICL2 186 GNAAETYLLQARIQAGACI48EQKVEDEEQCQ5Q expectations were different from ICL1 and PrpB of R. eutropha HF39 (Fig. 1A). No further ORF could be identified in the regions upstream (540 bp) or downstream (416 bp) of aceA2.

3.5. Heterologous expression of R. eutropha HF39 aceA1, aceA2 or aceB1 in E. coli

The aceA1 gene including 640 bp of the upstream and 480 bp of the downstream regions as well as the aceA2 gene including 320 bp of the upstream and 100 bp of the downstream regions were inserted into plasmid pBlueScript SK− and subsequently transformed into the E. coli ICL-negative mutant CGSC 5233 (aceA7Δ). In addition, aceB1 including 240 bp of the upstream and 300 bp of the downstream regions were inserted into plasmid pBlueScript SK− and subsequently transformed into the E. coli MSnegative mutant CGSC 5234 (aceB6Δ). The measured specific activities of ICL (70 and 19 U g−1, respectively) and of MS (52 U g−1) after 18 h growth of the transformants in LB medium at 37°C clearly showed that aceA1, aceA2 and aceB1 were functionally expressed in E. coli.

B. Malate synthases

(1) R. eutropha HF39 244 VLSTILAFEMDEELYEKRRHAGALAGWMDTIFY5X
(2) R. solanacearum 247 VLSTILAFEMDEELYEKRRHAGALAGWMDTIFY5X
(3) D. radiodurans 245 VLSTILAFEMDEELYEKRRHAGALAGWMDTIFY5X
(4) E. coli MSA 247 VLSTILAFEMDEELYEKRRHAGALAGWMDTIFY5X
(5) Streptomyces coelicolor 250 VLSTILAFEMDEELYEKRRHAGALAGWMDTIFY5X
(6) Vibrio cholerae 270 VLSTILAFEMDEELYEKRRHAGALAGWMDTIFY5X
(7) Z. daeguensis 258 VLSTILAFEMDEELYEKRRHAGALAGWMDTIFY5X
(8) Salmonella typhimurium 247 VLSTILAFEMDEELYEKRRHAGALAGWMDTIFY5X
(9) Aspergillus nidulans 238 VLSTILAFEMDEELYEKRRHAGALAGWMDTIFY5X
(10) Pichia angusta 240 VLSTILAFEMDEELYEKRRHAGALAGWMDTIFY5X
(11) Candida albicans 255 VLSTILAFEMDEELYEKRRHAGALAGWMDTIFY5X
(12) Candida tropicalis 255 VLSTILAFEMDEELYEKRRHAGALAGWMDTIFY5X
Consensus [K][R][K/G][D][E][N][Q][E]xEE[Q,E][I,K][R]HAGALAGWMDTIFY5X

Fig. 1. Signature patterns of ICL or methylisocitrate lyases (A) and MS (B). The conserved hexapeptide, [K/R][K/GH][KLMQR], which can be used as a signature pattern for ICL in A, and the conserved pattern [K/R][DENQ]HxxGxxDxxY[LIVM]F, which can be used as a signature for MS in B, are shown in bold font. A: (1) R. eutropha HF39 ICL1; (2) R. eutropha HF39 ICL2; (3) R. metallidurans CH34 Icl1p (http://genome.ornl.gov/microbial/reut/); (4) R. metallidurans CH34 Icl2p (http://genome.ornl.gov/microbial/reut/); (5) R. solanacearum Icl1p (NC_003295); (6) D. radiodurans Icl1p (NC_001263); (7) P. aeruginosa Icl1p (NC_002516); (8) E. coli Iclp (X12431); (9) M. tuberculosis Icl1p (NC_002755); (10) Corynebacterium glutamicum Icl1p (X75504); (11) R. eutropha HF39 PrpB (AAL03988). B: (1) R. eutropha HF39; (2) R. solanacearum (NC_003295); (3) D. radiodurans (NC_001263); (4) E. coli MSA (M19038); (5) Streptomyces coelicolor (AF206498); (6) Vibrio cholerae (NC_002505); (7) S. cerevisiae Mcl1p (NC_001146); (8) Aspergillus nidulans (NC_003197); (9) Pichia angusta (P21360); (10) Candida albicans (AF222907); (11) Candida tropicalis (D13415).

K(216), Q(217), C(218), G(219), H(220) and Q(221), whereas the amino acid residues Q(217) and Q(221) are different from ICL1 and PrpB of R. eutropha HF39 (Fig. 1A). No further ORF could be identified in the regions upstream (540 bp) or downstream (416 bp) of aceA2.

3.4. Characterization of the R. eutropha HF39 aceB1 gene

A 2636-bp nucleotide sequence of pHCaceB1 was analyzed, and the putative aceB1 gene encoding the MS of R. eutropha HF39, which is preceded by a ribosome binding site, was identified. The translational product consisted of 528 amino acid residues with a calculated molecular mass of 59 085 Da. The deduced amino acid sequence showed 84.6, 70.1, 56.9 or 17.3 mol% identity to MS1 (Mls1p) of R. solanacearum, R. metallidurans CH34 and Deinococcus radiodurans and to MS2 (Mls2p) of D. radiodurans, respectively. The consensus pattern of MS was found in MS1 of R. eutropha HF39 (Fig. 1B). A second incomplete ORF, ORF4*, encoding a putative haloacid dehalogenase-like hydrolase sharing 70.2 mol% identity to a putative 2-haloalkanoic acid dehalogenase of R. solanacearum was identified 68 bp upstream of aceB1. No further ORF was found in a region 509 bp downstream of aceB1. The vicinity of aceB1 in the genome of R. eutropha HF39 was similar to that of R. metallidurans CH34.

3.5. Heterologous expression of R. eutropha HF39 aceA1, aceA2 or aceB1 in E. coli

The aceA1 gene including 640 bp of the upstream and 480 bp of the downstream regions as well as the aceA2 gene including 320 bp of the upstream and 100 bp of the downstream regions were inserted into plasmid pBlueScript SK− and subsequently transformed into the E. coli ICL-negative mutant CGSC 5233 (aceA7Δ). In addition, aceB1 including 240 bp of the upstream and 300 bp of the downstream regions were inserted into plasmid pBlueScript SK− and subsequently transformed into the E. coli MS-negative mutant CGSC 5234 (aceB6Δ). The measured specific activities of ICL (70 and 19 U g−1, respectively) and of MS (52 U g−1) after 18 h growth of the transformants in LB medium at 37°C clearly showed that aceA1, aceA2 and aceB1 were functionally expressed in E. coli.

Interposon-mutants of the genes aceA1, aceA2 and aceB1 of R. eutropha HF39 were obtained as described, and the mutants R. eutropha HF39ΔaceA1, R. eutropha HF39ΔaceA2 and R. eutropha HF39ΔaceB1 were used for further analysis. For control, genomic DNA of all mutants was digested with HindIII, ligated into HindIII-linearized pBluescript SK− vector, and kanamycin-resistant clones were selected. The sequences of both kanamycin cassette flanking regions of R. eutropha HF39ΔaceA1, R. eutropha HF39ΔaceA2 and R. eutropha HF39ΔaceB1 were completely identical to those of aceA1, aceA2 and aceB1, respectively. This indicated that all target genes were correctly deleted in R. eutropha HF39.

Activities of ICL and MS in crude extracts of the aceA1, aceA2 or aceB1 interposon-mutants were measured and compared with those of the parent strain. Since growth of the mutants was much different from that of the parent strain, two-stage cultivation experiments were done. Cells were first grown in NB medium, harvested and washed and then transferred to MM containing acetate or gluconate. After 4 h cultivation, the cells were harvested and analyzed. In contrast to MS, expression of ICL was significantly induced by acetate in R. eutropha HF39. R. eutropha HF39ΔaceA1 grown on acetate revealed only 19% of ICL activity, while R. eutropha HF39ΔaceA2 retained 84% of the activity of the parent strain (Table 3). In addition, ICL was strongly induced by acetate in R. eutropha HF39ΔaceA2 suggesting that aceA1 is acetate inducible. Furthermore, the activities of MS in both R. eutropha HF39ΔaceA1 and R. eutropha HF39ΔaceA2 were about 20–30% lower (Table 3). However, in R. eutropha HF39ΔaceB1 grown on either carbon source, MS activity was about 60% lower in comparison to the parent strain R. eutropha HF39 (Table 3).

3.7. Disruption of glyoxylate bypass significantly affects growth on acetate as sole carbon source

To investigate whether mutations of aceA1, aceA2 or aceB1 affect the catabolism of acetate or gluconate, growth of the interposon-mutants was analyzed (Fig. 2). Cells were cultivated in NB medium, harvested, washed and afterwards resuspended in 50 ml MM supplemented with sodium acetate (0.5%, w/v) or sodium gluconate (0.5%, w/v). Cultivation was carried out at 120 rpm and 28°C for up to 96 h, and growth was recorded by measuring the increase of turbidity at 600 nm. R. eutropha HF39ΔaceA1 failed to grow on acetate. In contrast, R. eutropha HF39ΔaceA2 grew on acetate and gluconate similarly to the parent strain. R. eutropha HF39ΔaceB1 was able to grow on acetate and gluconate; however, its growth was significantly reduced on acetate.

To determine the possible effect of these mutations on growth on other carbon sources, the growth pattern was also examined using 0.2% (w/v) of the sodium salts of malate, lactate, fumarate, pyruvate, propionate, isocitrate or glyoxylate as sole carbon source. Reduced growth was only found for R. eutropha HF39ΔaceA1 and R. eutropha HF39ΔaceB1 when these mutants were cultivated on propionate, indicating that the glyoxylate bypass is in R. eutropha HF39 also required as anaplerotic reaction during the utilization of substrates which are catabolized via the 2-methylcitric acid cycle [11].

3.8. Effect of aceA1, aceA2 or aceB1 disruption on PHA accumulation

To examine the effect of mutations of aceA1, aceA2 or aceB1 on accumulation of PHA, R. eutropha HF39 and the respective mutants were grown in MM containing 0.5% (w/v) sodium gluconate or 0.5% (w/v) sodium acetate. The cells in the stationary phase were collected, lyophilized and the PHA content was analyzed by gas chromatography. Both R. eutropha HF39ΔaceA1 and HF39ΔaceA2 revealed a significantly lower poly(3HB) content while it was identical to that of the parent strain R. eutropha HF39 in R. eutropha HF39ΔaceB1 if gluconate was used as carbon source. During growth on acetate, the poly(3HB) content was not affected in R. eutropha HF39ΔaceA2 or R. eutropha HF39ΔaceB1 (Table 4).

Surprisingly, overexpression of aceA1 in R. eutropha

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activities (U g⁻¹ protein) in R. eutropha HF39 and the mutantsa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HF39</td>
</tr>
<tr>
<td>ICL</td>
<td></td>
</tr>
<tr>
<td>Gluconate</td>
<td>20.5 (3.5)</td>
</tr>
<tr>
<td>Acetate</td>
<td>73.2 (6.8)</td>
</tr>
<tr>
<td>MS</td>
<td></td>
</tr>
<tr>
<td>Gluconate</td>
<td>151.5 (21.6)</td>
</tr>
<tr>
<td>Acetate</td>
<td>158.1 (15.0)</td>
</tr>
</tbody>
</table>

*aCells were cultivated overnight in NB medium at 30°C and 120 rpm to the early stationary growth phase. The media contained 500 μg ml⁻¹ streptomycin for HF39 or 500 μg ml⁻¹ streptomycin and 160 μg ml⁻¹ kanamycin for mutants. Cells were then collected and washed, and the cell pellets were then suspended in MM supplemented with 0.5% (w/v) sodium gluconate or 0.5% (w/v) sodium acetate and incubated at 30°C for 4 h with gentle agitation. Data are means of triplicate determinations (± S.D. in parentheses).
VG12, a Tn5 mutant of *R. eutropha* HF39 defective in the *mdh* gene encoding a NADH-dependent malate dehydrogenase, did not only improve its growth on gluconate (data not shown) but also restored its poly(3HB) yield from gluconate as substrate (Table 4). Interestingly, *R. eutropha* VG12 exhibited only about one fourth of the specific activities of ICL, MS and NADPH-dependent isocitrate dehydrogenase. Levels of the wild-type were partly restored in the recombinant mutant harboring pBBR*aceA1* (data not shown). An explanation for this cannot be provided.

4. Discussion

In microorganisms, genes for ICLs and MSs involved in glyoxylate bypass show a great variability regarding number and organization. Whereas, for example, only one copy of ICL (*aceA*) and one copy of MS (*aceB*) exist in the archaeon *Haloferax volcanii* [13] and also in *R. solanacearum* [14], two copies for ICL (*ICL1* and *ICL2*) and for MS (*MLS1* and *MLS2*) exist in *Saccharomyces cerevisiae* [15]. Most microorganisms like *D. radiodurans* possess only one type of ICL [16], and the occurrence of ICL isoenzymes is rare. If two isoenzymes occur, the function of the second is mostly unknown. Like previously revealed by genome sequencing in *M. tuberculosis* (http://www.ncbi.nlm.nih.gov/PMGifs/Genomes/eub_g.html), *R. metallidurans* CH34, and *S. cerevisiae* (http://genome-www.stanford.edu/Saccharomyces/), two ICL isoenzymes were also found in *R. eutropha* HF39 in this study. Their arrangement on the three replicons [17] is different from that of *aceA1* or *aceA2* in *R. metallidurans* CH34, indicating that there are differences in the organization of genes in these phylogenetically related bacteria. In *R. eutropha* HF39, it is obvious that only ICL1 is specific to glyoxylate cycle since only *R. eutropha* HF39Δ*aceA1* failed to grow on acetate (Fig. 2). Reduced growth of mutants *R. eutropha* HF39Δ*aceA1* and HF39Δ*aceB1* on propionate may indicate an involvement of the glyoxylate cycle for provision of oxaloacetate during the function of the 2-methylcitric acid cycle like in *E. coli* [18].

Many microorganisms possess two distinct classes of MS which are encoded by different genes. In *E. coli*, *aceB* encodes the A-form and *gltB* the G-form. The

![Graph](image)

**Fig. 2.** Growth behavior of *aceA1*, *aceA2* and *aceB1* deletion mutants of *R. eutropha* HF39. Symbols: Δ*aceA1* mutant on gluconate (○) and on acetate (●); Δ*aceB1* mutant on gluconate (∆) and on acetate (▲); Δ*aceA2* mutant on gluconate (+) and on acetate (x); *R. eutropha* HF39 on gluconate (□) and on acetate (■).

**Table 4**

Accumulation of PHAs by *R. eutropha* HF39 and the *aceA1*, *aceA2*, *aceB1* or *mdh* mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Poly(3HB) content (% CDW)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Growth on gluconate</td>
</tr>
<tr>
<td><em>R. eutropha</em> HF39</td>
<td>72.4 (3.2)</td>
</tr>
<tr>
<td>HF39Δ<em>aceA1</em></td>
<td>57.0 (5.1)</td>
</tr>
<tr>
<td>HF39Δ<em>aceA2</em></td>
<td>58.6 (3.5)</td>
</tr>
<tr>
<td>HF39Δ<em>aceB1</em></td>
<td>72.4 (3.9)</td>
</tr>
<tr>
<td>VG12</td>
<td>58.4 (4.5)</td>
</tr>
<tr>
<td>VG12 (pBBR<em>aceA1</em>)</td>
<td>74.2 (1.6)</td>
</tr>
</tbody>
</table>

Cultivation was done in MM containing 0.5% (w/v) of the sodium salt of the indicated carbon source at 30°C and 120 rpm. After cell harvest in the stationary growth phase, poly(3HB) content was determined gas chromatographically in whole cells as described in Section 2. Data are means of triplicate determinations (±S.D. in parentheses). ND: these mutants do not grow on acetate; therefore, poly(3HB) contents could not be analyzed.
A-form is predominant in cells growing on acetate, while the G-form is mainly induced by glyoxylate [19]. Similarly, two different MS isoenzymes, Ms1p and Ms2p, were identified in *S. cerevisiae*. However, it is considered that only Ms1p contributes the activity both specific and limited to the glyoxylate cycle [15]. In addition, Ms1p is sensitive to carbon catabolite repression but nearly insensitive to nitrogen catabolite repression [15]. Also *D. radiodurans* possesses two Ms2s, which are encoded by aceB1 and aceB2 and located on two different chromosomes [16]. In *R. eutropha* HF39 like in *R. metallidurans* CH34 (http://genome.ornl.gov/microbial/reut/) only one gene encoding MS has been identified. The arrangement of aceB in the chromosome of *R. eutropha* HF39 is very similar to that of *R. metallidurans* CH34. It is well demonstrated that *R. eutropha* HF16 contains two circular chromosomes with sizes of 4.1 and 2.9 Mb in addition to a 0.44-Mb conjugative megaplasmid pHG1 [17]. Since *R. eutropha* HF39ΔaceB1 could still grow on acetate (Fig. 2) and still expressed about 40% of the MS activity found in the parent strain (Table 3), this may indicate that *R. eutropha* HF39 contains a second MS. However, a second gene encoding a protein homologous to MS could not be identified in the available (yet incomplete) *R. eutropha* genome sequence.

Disruption of the glyoxylate pathway by mutation of aceA in *P. putida* in addition to reduced flux through isocitrate dehydrogenase is predicted to increase the flux into de novo-fatty acid biosynthesis, and hence increase the amount of PHA<sub>mdh</sub> accumulated in the cells [20]. In contrast, disruption of either aceA gene resulted in *R. eutropha* HF39 in a significant decrease of the poly(3HB) content of the cells from gluconate. Furthermore, overexpression of aceA1 restored poly(3HB) yield in the Tn5-induced *mdh* mutant VG12 of *R. eutropha* HF39 from gluconate. Combining our results with data obtained from metabolic flux analysis for biosynthesis of poly(3HB) from various carbon sources [21] indicates that an intact TCA cycle and glyoxylate bypass are beneficial for cells to accumulate poly(3HB).

Acknowledgements

One of the authors (Z.X.W.) gratefully received a fellowship from the Deutscher Akademischer Austauschdienst (DAAD). This study was partially carried out within the framework of the Competence Network Göttingen ‘Genome research on bacteria’ (Genomik) financed by the German Federal Ministry of Education and Research (BMBF).

References


