

Identification of the 2-Methylcitrate Pathway Involved in the Catabolism of Propionate in the Polyhydroxyalkanoate-Producing Strain *Burkholderia sacchari* IPT101^T and Analysis of a Mutant Accumulating a Copolyester with Higher 3-Hydroxyvalerate Content

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Burkholderia sacchari IPT101^T induced the formation of 2-methylcitrate synthase and 2-methylisocitrate lyase when it was cultivated in the presence of propionic acid. The *prp* locus of *B. sacchari* IPT101^T is required for utilization of propionic acid as a sole carbon source and is relevant for incorporation of 3-hydroxyvalerate (3HV) into copolyesters, and it was cloned and sequenced. Five genes (*prpR*, *prpB*, *prpC*, *acnM*, and ORF5) exhibited identity to genes located in the *prp* loci of other gram-negative bacteria. *prpC* encodes a 2-methylcitrate synthase with a calculated molecular mass of 42,691 Da. *prpB* encodes a 2-methylisocitrate lyase. The levels of PrpC and PrpB activity were much lower in propionate-negative mutant IPT189 obtained from IPT101^T and were heterologously expressed in *Escherichia coli*. The *acnM* gene (ORF4) and ORF5, which are required for conversion of 2-methylcitric acid to 2-methylisocitric acid in *Ralstonia eutropha* HF39, are also located in the *prp* locus. The translational product of ORF1 (*prpR*) had a calculated molecular mass of 70,598 Da and is a putative regulator of the *prp* cluster. Three additional open reading frames (ORF6, ORF7, and ORF8) whose functions are not known were located adjacent to ORF5 in the *prp* locus of *B. sacchari*, and these open reading frames have not been found in any other *prp* operon yet. In summary, the organization of the *prp* genes of *B. sacchari* is similar but not identical to the organization of these genes in other bacteria investigated recently. In addition, this study provided a rationale for the previously shown increased molar contents of 3HV in copolyesters accumulated by a *B. sacchari* mutant since it was revealed in this study that the mutant is defective in *prpC*.

Polyhydroxyalkanoates (PHAs) are natural thermoplastics that occur in a wide variety of bacteria which synthesize these polyesters as carbon and energy storage compounds from renewable carbon sources. The thermoplastic properties of the copolyester poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [poly(3HB-co-3HV); Biopol] and its biodegradability are influenced mainly by the content of 3-hydroxyvalerate (3HV), which improves the properties of the molecule and also extends the range of its applications (21). Therefore, since PHAs can be obtained from renewable resources, commercial interest in these polyesters has increased a great deal during the last decade. Wild-type strains of different bacteria and mutants which are able to produce 3HV from unrelated carbon sources have been studied in the last few years (44, 45, 53).

The biotechnological processes now used for fermentative production of poly(3HB-co-3HV) by bacteria depend on a supply of propionic acid as a specific 3HV precursor substrate in addition to a carbohydrate or another main carbon source. This requirement allows workers to control the composition of

the polyester by changing the ratio of propionic acid to glucose (carbohydrate) in the medium (12). The strain which is used for PHA accumulation is an important factor in the production of poly(3HB-co-3HV). Many bacteria exhibit only a low 3HV yield with propionic acid ($Y_{3HV/PROP}$); in most cases this yield is approximately $0.1 \text{ g} \cdot \text{g}^{-1}$, whereas the theoretical value for the yield is $1.35 \text{ g} \cdot \text{g}^{-1}$ (17). The low yield may result from the predominance of oxidative pathways in propionic acid metabolism and the ability of the organisms to produce 3-hydroxybutyrate from this substrate. In fact, a number of pathways have been described for catabolism of propionic acid in different bacteria, and some of them seem to operate simultaneously (15, 56). The 2-methylcitric acid cycle (MCC) is one of these pathways, and at one time this pathway had been found only in yeasts and molds (31, 37). Recently, it was also found to occur in *Escherichia coli* and other non-PHA-producing microorganisms (16, 24, 50). The possibility that one or more oxidative propionic acid-degrading pathways operate in a PHA-producing strain could explain the low yield of 3HV in the copolyester.

Therefore, identification of the propionic acid catabolic pathways relevant for production of poly(3HB-co-3HV) in bacterial strains could improve such strains for industrial purposes. *Burkholderia sacchari* strain IPT101^T was isolated from the soil of a sugarcane plantation in Brazil, and a polyphasic

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taxonomic study identified this strain as a member of a novel species of the genus *Burkholderia* (7). Recent studies with propionic acid-negative mutants of this PHA-producing strain indicated that propionic acid is catabolized by at least two pathways, an α -oxidation pathway and an alternative pathway that has not been identified yet. These two pathways could be responsible for more than 80% of the propionic acid utilization under PHA accumulation conditions and could account for the overall propionate catabolism (41). Mutant IPT189, which was unable to grow on propionic acid as a sole carbon source but still incorporated 3HV units from this substrate into the copolyester, was not affected in the α -oxidation pathway (41). This mutant exhibited an improved $Y_{3HV/PROP}$, which ranged from 0.80 to 1.20 g · g⁻¹ depending on the cultivation strategy used to produce poly(3HB-co-3HV) from sucrose and propionic acid (41). This study was performed to identify the genes in this mutant that are affected and to clarify propionic acid catabolism in *B. sacchari* IPT101^T.

MATERIALS AND METHODS

Bacterial strains and plasmids. Wild-type strain *B. sacchari* IPT101^T (Suc⁺ Prp⁺) (7, 17) and mutant IPT189 (Suc⁺ Prp⁻) obtained from IPT101^T (41) were studied. *E. coli* S17-1 (42) and cosmids pVK100 (27) and pHC79 (20) were used in cloning experiments. *E. coli* XL-1 Blue (11) and the phagemids pBluescript SK⁻ and pBCSK⁺ (Stratagene) were used for DNA sequencing. The propionate-negative Tn5 mutants SK 7286, VG17, and P2 of *Ralstonia eutropha* HF39 (8) were employed for phenotypic complementation.

Media and growth conditions. Cells of *E. coli* S 17-1 and XL-1 Blue were cultivated in Luria-Bertani (LB) medium at 37°C; isopropyl- β -D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) were added if appropriate (38). Strains of *B. sacchari* and *R. eutropha* were grown at 30°C in either nutrient broth or mineral salts medium (MM) (39) supplemented with carbon sources as indicated below. Solid media contained 15 to 20 g of agar per liter. Antibiotics were added to the media at the following final concentrations, if necessary: tetracycline, kanamycin, and ampicillin, 15, 50, and 100 μ g · ml⁻¹, respectively, for recombinant strains of *B. sacchari*; and kanamycin, streptomycin, and tetracycline, 160, 500, and 25 μ g · ml⁻¹, respectively, for mutants or recombinant strains of *R. eutropha*.

Isolation of DNA, transformation, and electrophoretic methods. Genomic DNA and cosmids pVK100 and pHC79 were extracted by the methods described by Ausubel et al. (2), Marmur (29), and Birnboim and Doly (4), respectively. Transformation protocols, other molecular techniques, and agarose gel electrophoresis were performed as described by Sambrook et al. (38), unless indicated otherwise. DNA fragments were isolated from agarose gels with a Gene Clean kit (54). Enzymes, reagents, and kits were used according to the instructions provided by the manufacturers.

Construction of a genomic library of *B. sacchari* IPT101^T. Partially *EcoRI*-digested or completely *HindIII*-digested genomic DNA was ligated to *EcoRI*-restricted cosmid pVK100 or to *HindIII*-restricted cosmid pHC79 DNA. The ligation mixtures were packaged in λ phages and transduced to *E. coli* S17-1, and clones were selected on LB medium containing tetracycline. Propionic acid-negative mutant IPT189 of *B. sacchari* IPT101^T (recipient) and *E. coli* S17-1 harboring hybrid plasmids (donor) were conjugated by spot agar mating, and transconjugants were selected on MM agar containing sucrose (0.1%, wt/vol) as the sole carbon source plus tetracycline.

DNA-DNA hybridization. Southern hybridization was carried out by the method described by Oelmüller et al. (32) at 68°C. Colony hybridization was performed by the method of Grunstein and Hogness (18) at 68°C.

PCR amplification. PCR amplification of DNA encoded on plasmids or genomic DNA was carried out as described in a laboratory manual (38) by using VENT_R DNA polymerase (New England Biolabs, Schwalbach/Taunus, Germany) or Platinum Pfx DNA polymerase (Gibco BRL Life Technologies, Karlsruhe, Germany) and an Omnigene HBTR3CM DNA thermal cycler (Hybaid, Heidelberg, Germany).

Determination of DNA sequence. DNA fragments of interest were ligated to the phagemid pBluescript SK⁻ and transformed into competent cells of *E. coli* XL-1 Blue. Clones containing inserted DNA were selected on LB medium containing X-Gal and IPTG and cultivated in liquid LB medium with ampicillin

and tetracycline, and hybrid plasmids were extracted in order to sequence the fragments. The primer-hopping strategy (47) was used to determine the DNA sequence of both DNA strands. Sequencing was performed by using a Sequi Therm EXCEL TM II long-read cycle sequencing kit (Epicentre Technologies, Madison, Wis.) and IRD 800-labeled oligonucleotides (MWG-Biotech, Ebersberg, Germany). Sequencing was performed with a LI-COR 4000L automatic sequencing apparatus (MWG-Biotech).

Sequence data analysis. Sequence data were compared with sequences in the GenBank and Prosite databases by using the programs BlastSearch 2.0.10 (1) and DBGET (3). A dendrogram was constructed with the program ClustalW (51).

Preparation of crude extracts. Cells from 50-ml cultures were washed in 66.7 mM potassium phosphate buffer (pH 6.9) and resuspended in 5 ml of this buffer, and 10 μ g of DNase I was added. The cells were disrupted by sonication with a Bandelin sonopuls GM200 (Bandelin Electronic, Berlin, Germany) by using an amplitude of 16 μ m (1 min/ml). During ultrasonication the samples were chilled in an NaCl-ice bath. Soluble protein fractions of the crude extracts were obtained by centrifugation for 5 min at 13,000 rpm and 4°C (Minifuge; Heraeus, Osterode, Germany).

Cloning of *prpB* and *prpC* and heterologous expression in *E. coli*. We constructed oligonucleotides to amplify *prpB* (*prpBUP* [5'-AAAGGATCCTTTGG AGACTCGCACACATGAACAG-3']) and *prpBRP* [5'-AAAGAATTGCTTAC TTCTCCGCGGCGAAC-3']) and *prpC* (*prpCUP* [5'-AAATCTAGAGAGAG AAAGGACGACACATGAG-3']) and *prpCRP* [5'-AAACTGCAGATGGCGG TGCCCTGCATCAG-3']) from genomic DNA of *B. sacchari* IPT101^T. The 920-bp DNA fragment obtained with *prpBUP* and *prpBRP* was cloned into pBluescript SK⁻, which resulted in pSK⁻/*prpB*_{BS}. *prpCUP* contained an *XbaI* recognition sequence and *prpCRP* contained a *PstI* recognition sequence; this enabled forced cloning of *prpC* downstream of and colinear to the *lacZ* promoter with *XbaI/PstI*-restricted pBluescript SK⁻ DNA, which resulted in pSK⁻/*prpC*_{BS}.

Cells of *E. coli* harboring pSK⁻/*prpB*_{BS}, pSK⁻/*prpC*_{BS}, or pBluescript SK⁻ were grown at 30°C for 16 h in 50 ml of LB medium containing 75 μ g of ampicillin per ml plus 0.5 mM IPTG. The cells were harvested by centrifugation in a Minifuge RF (Heraeus, Osterode, Germany) (10 min, 4,500 rpm, 4°C), washed with 100 mM Tris-HCl (pH 8.0), resuspended in 5 ml of 100 mM Tris-HCl, and disrupted by two passages through a French press.

Determination of the activities of the MCC enzymes. The activity of 2-methylcitrate synthase (EC 4.1.3.31) was measured by the method of Srere (43). The cuvette used (diameter, 1 cm) contained 1 ml (total volume) of a solution containing 2 mM oxaloacetate, 250 μ M propionyl coenzyme A (propionyl-CoA), and 2 mM 5,5'-dithiobis-(2-nitrobenzoate) in 66.7 mM potassium phosphate buffer (pH 6.9). After addition of the crude extract, the increase in absorbance at 412 nm ($\epsilon = 13.6 \text{ cm}^2 \cdot \mu\text{mol}^{-1}$) was determined with an Ultraspec 2000 photometer (Pharmacia, Uppsala, Sweden).

The activity of 2-methylisocitrate lyase was assayed in a 0.5-ml (total volume) mixture 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 adding 1.25 mM K₃-methylisocitrate, which was obtained by alkaline cleavage of the methylisocitric acid lactone (10). The increase in absorbance at 324 nm was measured. The molar extinction coefficient of pyruvate phenylhydrazone was 12 mM⁻¹ · cm⁻¹ (28).

One unit of enzyme activity was defined as the amount of activity that converted 1 μ mol of substrate per min. The amount of soluble protein was determined by the method of Bradford (6); crystalline bovine serum albumin was used as the standard.

Nucleotide sequence accession number. The nucleotide and amino acid sequence data determined in this study have been deposited in the GenBank nucleotide sequence database under accession no. AY033092.

RESULTS

Complementation of a propionate-negative mutant. UV-induced mutant IPT189 of *B. sacchari* IPT101^T is not able to grow on propionic acid as a sole carbon source but still incorporates 3HV into PHA when it is starved for nitrogen (41). We decided to use this mutant for identification of the genes required for propionate catabolism in *B. sacchari* by phenotypic complementation. Four clones of the gene library prepared from *EcoRI*-restricted genomic DNA of IPT101^T and pVK100 were identified by minicomplementation of mutant IPT189. In these clones the ability to grow on propionic acid was restored.

The hybrid plasmid of one clone (p17227), which consisted of four different *EcoRI* restriction fragments, was digested with *EcoRI*, and the fragments were ligated to *EcoRI*-digested pVK100 DNA. The ligation products were transformed into *E. coli* S 17-1, and clones were selected on LB agar plates containing tetracycline. Phenotypic complementation of IPT189 resulted in identification of six transconjugants in which the Prp⁺ phenotype was restored. All hybrid plasmids harbored a 2-kbp *EcoRI* fragment; four plasmids (pB₆, pB₁₀, pB₂₇, and pC₁₄) harbored only this fragment, whereas one (pB₇) also harbored a 3-kbp *EcoRI* fragment and one (pA₂₆) also harbored the 3-kbp fragment plus a 5-kbp *EcoRI* fragment. When the 2-kbp *EcoRI* fragment was digested with *SalI*, a 1.1-kbp *SalI* restriction fragment was obtained, and this 1.1-kbp fragment was ligated to *SalI*-digested pVK100 DNA, which still complemented mutant IPT189. Analysis of the 2-kbp *EcoRI* fragment revealed partial sequences of two open reading frames, whose putative translational products exhibited high levels of similarity to the 2-methylcitrate synthases (*prpC* gene product) of *E. coli* (50) and *Salmonella enterica* (24) or to aconitate hydratases of several organisms (see below).

Characterization of the *Burkholderia prp* locus. In order to clone a larger DNA fragment comprising the entire *prp* region, genomic DNA of *B. sacchari* IPT101^T was digested with different restriction enzymes and used for Southern hybridization with the 2-kbp *EcoRI* fragment as a probe. Fragments that were approximately 25 to 30 kbp long (*HindIII*-digested DNA), 20 kbp long (*Bam*HI-digested DNA), and 7 kbp long (*Cla*I-digested DNA) gave hybridization signals. Subsequently, *HindIII*-digested DNA was ligated to *HindIII*-linearized pHC79 and transduced into *E. coli* S17-1. Tetracycline-resistant clones were screened by colony hybridization by using the 2-kbp *EcoRI* fragment as a homologous probe. Five positive clones (clones A to E), each harboring a 25.5-kbp *HindIII* fragment, were identified (Fig. 1). The hybrid cosmid of clone D, which harbored only the 25.5-kbp *HindIII* fragment, was restricted with *EcoRI*, which resulted in eight subfragments (1.4, 2, 2.4, 2.9, 3.8, 4, 6.5 [pHC79], and 9 kbp). All of these fragments were subcloned into the vector pBCSK⁺ and were at least partially sequenced. The 2-kbp *EcoRI* fragment was identical to the fragment that was initially cloned, whereas the 2.9-kbp fragment was adjacent to and upstream of the 2-kbp *EcoRI* fragment and the 9-kbp fragment was adjacent to and downstream of the 2-kbp *EcoRI* fragment (Fig. 2A). The 4-kbp *EcoRI* fragment was localized upstream of the 2.9-kbp fragment (Fig. 2A). On the last four fragments five open reading frames were identified, and these open reading frames exhibited similarities to MCC structural genes (Fig. 2B).

ORF2 encodes a 2-methylisocitrate lyase (PrpB). The 892-bp open reading frame ORF2 starts with an ATG codon at nucleotide 2140 that is the most probable translational initiation codon of the structural gene which was designated *prpB*. This was concluded on the basis of the amino acid alignment and on the basis of the tentative ribosome-binding site which preceded this ATG. The *prpB* gene product consisted of 297 amino acids, and it had a calculated molecular mass of 31,732 Da and a pI of 4.99. The primary structure of PrpB revealed a level of identity of 64 mol% to the *prpB* gene product of *R. eutropha* HF39 (Table 1), which catalyzes cleavage of 2-methylisocitric acid to succinate and pyruvate in this strain (8).

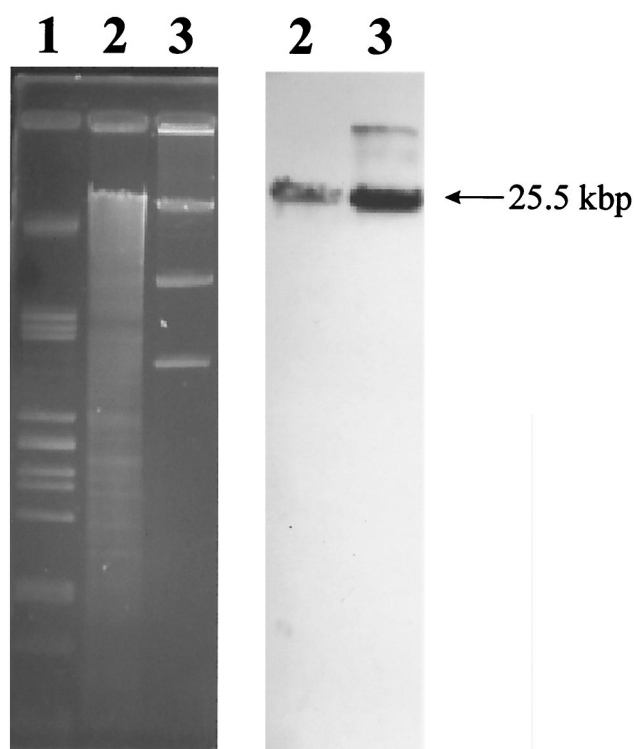


FIG. 1. Southern hybridization of *E. coli* S17-1 clones, harboring *HindIII*-digested genomic DNA of *B. sacchari* IPT101^T in cosmid pHC79, identified by colony hybridization by using the 2-kbp *EcoRI* fragment as a probe. Lane 1, *Pst*I-digested λ DNA; lanes 2, *HindIII*-digested genomic DNA of *B. sacchari* IPT101^T; lanes 3, *HindIII*-restricted hybrid cosmids of the *E. coli* S17-1 clone.

Furthermore, an isocitrate lyase signature (K-[KR]-C-G-H-[LMQR]) was present, as it is in all known methylisocitrate lyase sequences; this signature includes highly conserved cysteine (C), glutamate (G), and histidine (H) residues which are important for the catalytic activity of isocitrate lyases (<http://www.expasy.ch/cgi-bin/nicesite.pl?PS00161>).

To confirm the presence of a 2-methylisocitrate lyase in *B. sacchari*, cells of *B. sacchari* IPT101^T and IPT189 were grown in 50 ml of MM containing 1.0% (wt/vol) sodium gluconate for 30 h at 30°C. The cells were then harvested, washed with sterile saline, and transferred into fresh MM containing 0.2% (wt/vol) sodium propionate (IPT189 and IPT101^T) or 0.2% (wt/vol) sodium gluconate (IPT101^T) as the sole carbon source. After 24 h of incubation at 30°C, we measured the 2-methylisocitrate lyase activities in crude extracts of the propionate-induced and uninduced cells of IPT101^T and the propionate-induced cells of IPT189, as shown in Table 2. The results revealed that formation of 2-methylisocitrate lyase is induced by propionic acid in *B. sacchari* IPT101^T, as shown previously for the MCC in *R. eutropha* HF39 (8). The activity of this enzyme was strongly reduced in the propionic acid-negative mutant IPT189. Furthermore, a 2-methylisocitrate lyase activity of 16.6 mU/mg of protein was observed in the recombinant *E. coli* (pSK⁻/*prpB*_{Bs}), compared to an activity of only 2.6 mU/mg of protein in an *E. coli* strain harboring pBluescript SK⁻ as a control.

Thirty-four nucleotides upstream of *prpB* a consensus σ^{54}

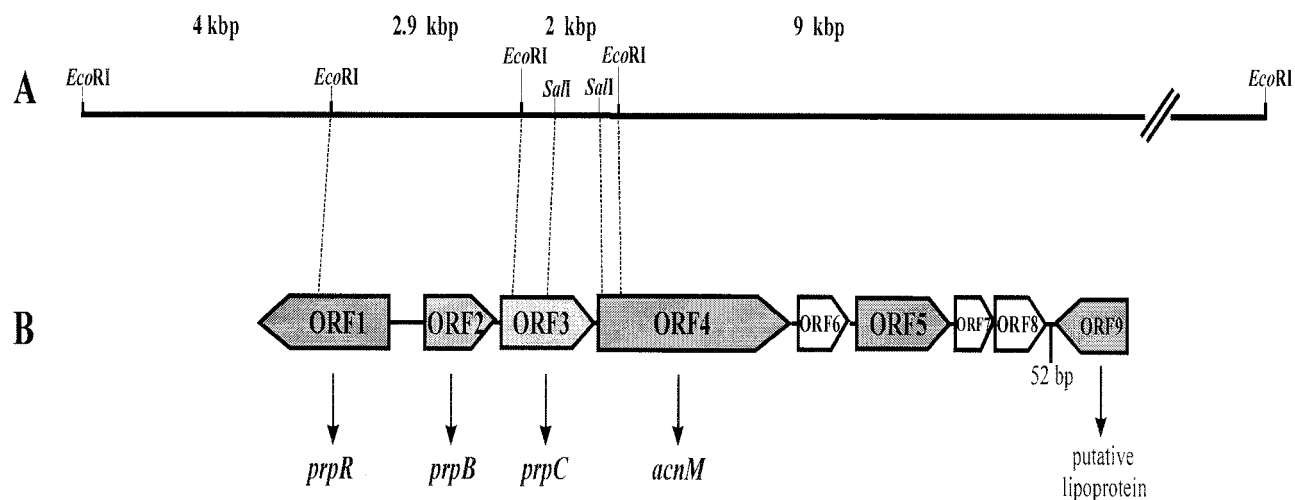


FIG. 2. (A) Arrangement of four *EcoRI* subfragments on the 25.5-kbp *HindIII* fragment of *B. sacchari* IPT101^T; (B) organization of the *prp* locus in *B. sacchari* IPT101^T.

promoter like that in *S. enterica* serovar Typhimurium LT2 (33) and a putative upstream activator sequence (**GATTTCAAATTTGAAATCNCAATTCAGACAGGAAATTG** [the different typefaces and underlining indicate the sequences which correspond]) like that in *R. eutropha* (8) were identified. The putative translational product of ORF1 exhibited 37 mol% amino acid identity to the *prpR* gene product of *Pseudomonas aeruginosa* and to other transcriptional activators belonging to the σ^{54} (*rpoN*) family (33). The molecular mass of PrpR,

70,598 Da, corresponded to the molecular mass of the putative *prpR* gene product of *R. eutropha* (8). It is therefore probably a transcriptional activator of the *prp* operon in *B. sacchari* and is referred to in this paper as *prpR*.

ORF3 encodes a 2-methylcitrate synthase (PrpC). The deduced amino acid sequence of ORF3 (length, 1,167 bp), which started with the ATG at position 3082, exhibited levels of identity ranging from 51 mol% (*Vibrio cholerae*, *Legionella pneumophila*) to 88 mol% (*Burkholderia cepacia*) to *prpC* gene products of several organisms (Table 1) and had a molecular mass of 42,691 Da and a theoretical pI of 7.27. The *prpC* gene encodes a 2-methylcitrate synthase in *R. eutropha* HF39 (8), *E. coli* (50), and *S. enterica* serovar Typhimurium (22). A comparison of the amino acid sequences of all of the available *prpC* translational products of several organisms resulted in identification of two motifs with 100% identity to the citrate synthase signature (<http://www.expasy.ch/cgi-bin/prosite-search-ac?PS00480>) and to the putative active sites of citrate synthases, both of which are highly conserved in all putative methylcitrate synthases (Fig. 3).

To confirm the presence of a 2-methylcitrate synthase in *B. sacchari*, cells were grown in the way described above for the analysis of the 2-methylisocitrate lyase. Measurement of 2-methylcitrate synthase activity revealed that formation of this enzyme was also induced by propionic acid, and cells of mutant

TABLE 1. Comparison of the translational products of genes located in the *prp* cluster of several organisms with the translational products of *prpB*, *prpC*, *acnM*, and ORF5 of *B. sacchari* IPT101^T

Strain	mol% identity with products of <i>B. sacchari</i> IPT101 ^T genes ^a				Reference ^b
	<i>prpB</i>	<i>prpC</i>	<i>acnM</i>	ORF5	
<i>R. eutropha</i> HF39	64	82	91	84	8
<i>B. cepacia</i>	ND ^c	88	83	80	
<i>N. meningitidis</i> serogroup A	63	74	79	67	34
<i>N. meningitidis</i> serogroup B	63	74	78	67	49
<i>P. aeruginosa</i> PAO1	61	54	83	71	46
<i>P. putida</i> KT2440	64	53	86	83	
<i>P. fluorescens</i>	60	54	84	77	
<i>P. syringae</i>	64	52	— ^d	79	
<i>E. coli</i> K-12	58	76	ND	ND	50
<i>E. coli</i> O157:H17	57	76	ND	ND	35
<i>S. enterica</i> serovar Typhimurium	58	75	ND	ND	22
<i>L. pneumophila</i>	ND	51	ND	ND	
<i>V. cholerae</i>	57	51	75	69	19
<i>S. putrefaciens</i>	66	54	84	80	
<i>R. metallidurans</i> CH34	60	84	89	80	

^a Levels of amino acid identity.

^b The data for some of the organisms were obtained from the following sources: *B. cepacia*, *P. fluorescens*, and *R. metallidurans*, <http://www.expasy.ch/cgi-bin/nicesite.p1?PS00161>; *P. putida*, *P. syringae*, and *S. putrefaciens*, <http://www.tigr.org/cgi-bin/BlastSearch/blast.cgi?organism>; and *L. pneumophila*, accession no. AAD42885.

^c ND, not detected in the database.

^d —, only incomplete sequences were available.

TABLE 2. Activities of 2-methylcitrate lyase and 2-methylcitrate synthase in crude extracts of *B. sacchari* wild-type strain IPT101^T and mutant Prp189

Crude extract	Carbon source ^a	Sp act (U/mg of protein)	
		2-Methylcitrate synthase	2-Methylisocitrate lyase
IPT101 ^T	Sodium gluconate	0.076	0.017
	Sodium propionate	0.410	0.112
Prp189	Sodium propionate	0.076	0.006

^a The cells were cultivated on MM containing different carbon sources after growth in MM containing sodium gluconate.

271 GFGH P V Y T I S D P R 283	321 F P N L D W F S A 329	<i>B. sacchari</i> IPT101 ¹
271 GFGH P V Y T I S D P R 283	321 F P N L D W F S A 329	<i>B. cepacia</i> (contig261)
267 GFGH P V Y T T G D P R 279	317 F P N L D W F S A 325	<i>R. eutropha</i> HF39 (8; AF325554)
267 GFGH P V Y T I S D P R 279	317 F P N L D W F S A 325	<i>R. metallidurans</i> CH34 (contig371)
267 GFGH P V Y T I S D P R 279	317 F P N L D W F S A 325	<i>N. meningitidis</i> serogroup A (34; NP_284754)
267 GFGH P V Y T I S D P R 279	317 F P N L D W F S A 325	<i>N. meningitidis</i> serogroup B (49; A81200)
258 GFGH A I Y K E S D P R 270	308 F P N A D F Y H A 316	<i>P. putida</i> KT2440 (contig10768)
258 GFGH A I Y K D S D P R 270	308 F P N A D F Y H A 316	<i>P. aeruginosa</i> PAO1 (46; B83547)
258 GFGH A I Y K D S D P R 270	308 F P N A D F Y H A 316	<i>P. fluorescens</i> (contig267)
258 GFGH A I Y K E S D P R 270	308 F P N A D F Y H A 316	<i>P. syringae</i> (contig5376)
259 GFGH A I Y R E S D P R 271	309 F C N A D F F H A 317	<i>V. cholerae</i> (19; A82213)
271 GFGH P V Y T I A D P R 283	321 F P N L D W F S A 329	<i>E. coli</i> K12 (50; P31660)
271 GFGH P V Y T I A D P R 283	321 F P N L D W F S A 329	<i>E. coli</i> O157:H17 (35; NP_286073)
271 GFGH P V Y T I A D P R 283	321 F P N L D W F S A 329	<i>S. enterica</i> serovar Typhimurium (22; Q56063)
254 GFGH R V Y T T C D P R 266	304 F P N L D F Y S A 312	<i>L. pneumophila</i> (AAD42885)
258 GFGH A I Y R D S D P R 270	308 F C N A D F F H A 316	<i>S. putrefaciens</i> (contig93)
343 G Y G H G V L R K P D P R 355	399 H P N V D A A S G 407	<i>A. nidulans</i> (9)

FIG. 3. Comparison of 2-methylcitrate synthase motifs with the corresponding citrate synthase signature. The shaded amino acids are the putative active sites (http://www.jgi.doe.gov/tempweb/JGI_microbial/html/index.html), which were identified on the basis of similarity. References, accession numbers (if available) and contig numbers are given in parentheses. Data for some of the organisms were obtained from the following websites: *B. cepacia*, *R. metallidurans* CH34, and *Pseudomonas fluorescens*, <http://www.expasy.ch/cgi-bin/nicesite.pl?PS00161>; and *P. putida* KT2440, *P. syringae*, and *S. putrefaciens*, <http://www.tigr.org/cgi-bin/BlastSearch/blast.cgi?organism>.

IPT189 exhibited much less activity of this enzyme than the wild type when they were cultivated in the presence of propionic acid (Table 2). Furthermore, a level of 2-methylcitrate synthase activity of 0.91 U/mg of protein was determined in recombinant *E. coli*(pSK⁻/prpC_{Bs}), compared to a level of activity of 0.006 U/mg of protein in an *E. coli* strain harboring only pBluescript SK⁻.

In addition, the *prpC* gene of *R. eutropha* (8) was ligated into the vector pBRR1-JO2 (J. Overhage, personal communication) and transformed into *E. coli* XL-1 Blue. Hybrid plasmids pBRR1-JO2/*prpC*_{Re} and pBRR1-JO2 were then transferred via *E. coli* S17-1 by conjugation into *B. sacchari* propionate-negative mutant IPT189. Hybrid plasmid pBRR1-JO2/*prpC*_{Re} restored the ability of mutant IPT189 to grow on MM agar plates containing 0.1% (wt/vol) sodium propionate as the sole carbon source, whereas IPT189 harboring only the vector did not grow.

Putative gene product of ORF4. Downstream and 136 bp from ORF3, ORF4 started with a GTG codon at position 4385, which was preceded by a putative ribosome-binding site. The putative translational product of ORF4 had a calculated molecular mass of 94,376 Da and a theoretical pI of 5.27. A comparison of the deduced amino acid sequence of ORF4 with the primary structures of other proteins revealed that the highest levels of identity, 91 and 89 mol%, were the levels of identity to the aconitate hydratases of *R. eutropha* HF39 (8)

and *Ralstonia metallidurans* CH34 (http://www.jgi.doe.gov/tempweb/JGI_microbial/html/index.html), respectively, as shown in Table 1; the genes for these enzymes are located downstream of the *prpC* gene in a cluster (8). In contrast, the levels of identity of the gene product of ORF4 to aconitases in the tricarboxylic acid cycle were rather low (40 to 45 mol%). A dendrogram based on the amino acid sequences of several aconitate hydratases revealed that the putative *acnM* translational products form a cluster, which separates them from citric acid cycle aconitases (Fig. 4). 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 occur in all known *acnM* gene products, as shown in Fig. 5. A difference in the vicinity of these cysteine residues was found when the *acnM* translational products and the other aconitate hydratases were compared. Whereas all *acnM* translational products have a polar asparagine residue next to the cysteine residue (Fig. 5), in most cases the other aconitate hydratases have the nonpolar amino acid isoleucine at this position; in some cases an enzyme has valine or leucine at this position (<http://www.expasy.ch/cgi-bin/prosite-search-ac?PS00450>; <http://www.expasy.ch/cgi-bin/prosite-search-ac?PS01244>).

Complementation of Tn5-induced *prpC* and *acnM* mutants of *R. eutropha* HF39. Phenotypic complementation of Tn5-

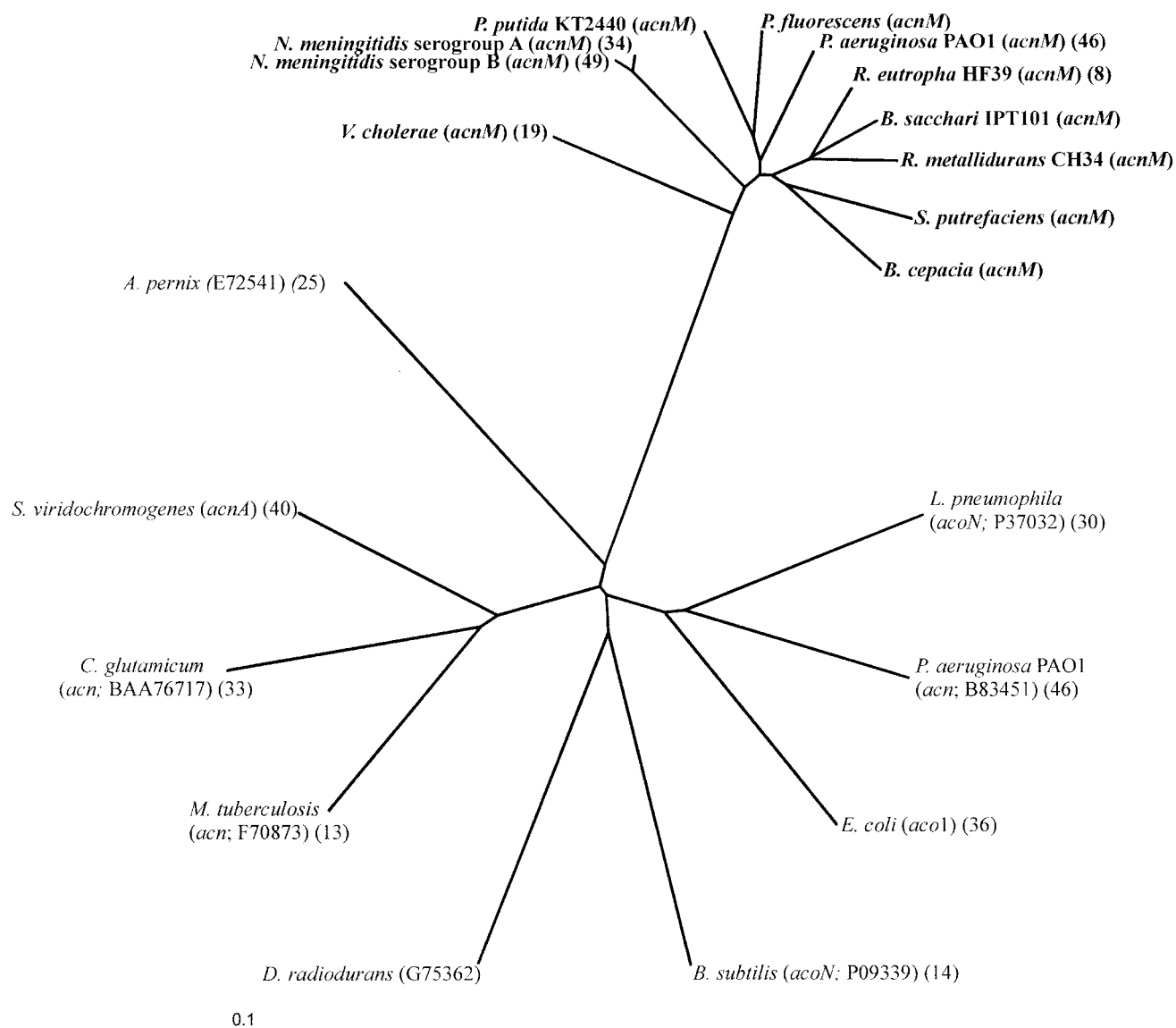


FIG. 4. Dendrogram based on aconitases and *acnM* translational products of several bacteria. The numbers in parentheses are accession numbers and references. Data for some of the organisms were obtained from the following websites: *P. fluorescens* and *B. cepacia*, <http://www.expasy.ch/cgi-bin/nicesite.pl?PS00161>; and *S. putrefaciens* and *P. putida*, <http://www.tigr.org/cgi-bin/BlastSearch/blast.cgi?organism>.

induced mutants VG17 and P2 of *R. eutropha* HF39 with a defect in a putative aconitate hydratase gene and phenotypic complementation of *prpC* mutant SK7286 of *R. eutropha* HF39 with the 2-kbp *EcoRI* or 1.1-kbp *SalI* fragment in cosmid pVK100 failed. Since neither fragment harbored the complete *prpC* gene or the complete aconitate hydratase gene, complementation of *B. sacchari* mutant IPT189 most probably resulted from homologous recombination of the incomplete genes present on the plasmids with the corresponding defective parts in the mutant genome, a process that can occur only between highly homologous or identical DNA sequences. Therefore, no complementation of the *R. eutropha* mutants could occur.

Putative gene product of ORF5. The 396-amino-acid translational product of 1,191-bp ORF5 had a calculated molecular mass of 41,443 Da and a theoretical pI of 5.95 and exhibited

the highest levels of identity (84 and 83 mol%) to the ORF5 translational products of *R. eutropha* HF39 (8) and *Pseudomonas putida* KT2440 (Table 1). Analysis of mutants has shown previously that in *R. eutropha* HF39 ORF5 is required for conversion of 2-methylcitrate into 2-methylisocitrate (8).

Putative gene products of ORF6, ORF7, ORF8, and ORF9. Three additional small open reading frames that are 366 bp long (ORF6), 198 bp long (ORF7), and 372 bp long (ORF8) are located in the *prp* cluster of *B. sacchari* IPT101^T. The translational product of ORF6 exhibited 50 mol% identity to a hypothetical 14.0-kDa protein encoded in the *fabI-sapF* region of *E. coli* (5), whereas the translational products of ORF7 and ORF8 exhibited 53 and 44 mol% identity to hypothetical proteins Rv1113 and Rv1114 of *Mycobacterium tuberculosis*, respectively (13). ORF9 was located 52 bp downstream of and antilinear with respect to the *prp* locus, and the translational

402 AITSC [†] TNTSNPR ⁴¹³	464 GFGIVAFAC [†] TT [†] CNG ⁴⁷⁷	<i>B. sacchari</i> IPT101 ^T	<i>acnM</i> translational products
402 AITSC [†] TNTNNPR ⁴¹³	464 GFGVVAYAC [†] TS [†] ONG ⁴⁷⁷	<i>B. cepacia</i> (contig359)	
407 AITSC [†] TNTSNPR ⁴¹⁸	469 GFGIVAFAC [†] TT [†] CNG ⁴⁸²	<i>R. eutropha</i> HF39 (8; AF325554)	
402 AITSC [†] TNTSNPR ⁴¹³	464 GFGIVAFAC [†] TT [†] CNG ⁴⁷⁷	<i>R. metallidurans</i> CH34 (contig371)	
408 AITSC [†] TNTSNPR ⁴¹⁹	470 GFGIVAFAC [†] TT [†] CNG ⁴⁶³	<i>N. meningitidis</i> serogroup A (34; H81775)	
408 AITSC [†] TNTSNPR ⁴¹⁹	470 GFGIVAFAC [†] TT [†] CNG ⁴⁶³	<i>N. meningitidis</i> serogroup B (49; C81200)	
403 AITSC [†] TNTSNPR ⁴¹⁴	465 GFGIVAFAC [†] TT [†] CNG ⁴⁷⁸	<i>P. putida</i> KT2440 (contig10768)	
407 AITSC [†] TNTSNPR ⁴¹⁸	465 GFGVVAYAC [†] TS [†] ONG ⁴⁸²	<i>P. aeruginosa</i> PAO1 (46; A83547)	
404 AITSC [†] TNTSNPR ⁴¹⁵	465 GFGIVAFAC [†] TT [†] CNG ⁴⁷⁸	<i>P. syringae</i> (contig5376)	
402 AITSC [†] TNTSNPR ⁴¹³	464 GFGVVAYAC [†] TT [†] CNG ⁴⁷⁷	<i>P. fluorescens</i> (contig267)	
411 AITSC [†] TNTSNPR ⁴²²	473 GFGIVAYAC [†] TT [†] CNG ⁴⁸⁶	<i>V. cholerae</i> (19; B82213)	
402 AITSC [†] TNTSNPR ⁴¹³	464 GFGIVGFA [†] TT [†] CNG ⁴⁷⁷	<i>S. putrefaciens</i> (contig93)	

431 AITSC [†] TNTSNPS ⁴⁴²	493 GFNLVGYG [†] CT [†] TCIG ⁵⁰⁶	<i>E. coli</i> (<i>aco1</i>) (36; S22375)	aconitases acting in the
431 AITSC [†] TNTSNPS ⁴⁴²	493 GFNLVGYG [†] CT [†] TCIG ⁵⁰⁶	<i>E. coli</i> O157:H17 (<i>acnA</i>) (35; AE005379)	tricarboxylic acid cycle
468 AITSC [†] TNTSNPY ⁴⁷⁹	530 GFNLVGYG [†] CT [†] TCIG ⁵⁴²	<i>Streptomyces viridochromogenes</i> (<i>acnA</i>) (40; Y17270)	
442 AITSC [†] TNTSNPY ⁴⁵³	504 GFNLVGYG [†] CT [†] TCIG ⁵¹⁷	<i>B. halodurans</i> (<i>citB</i>) (48; BAB06018)	
439 AITSC [†] TNTSNPS ⁴⁵⁰	507 GFNLVGF [†] CT [†] TCIG ⁵¹⁴	<i>Bradyrhizobium japonicum</i> (<i>acoN</i>) (52; P70920)	

FIG. 5. Comparison of motifs of the *acnM* translational products of several organisms with the aconitase signature. The shaded amino acids are the putative cysteine residues which bind the Fe-S-cluster (identified on the basis of similarity) (<http://www.tigr.org/cgi-bin/BlastSearch/blast.cgi?organism>). The boldface N is a highly conserved asparagine residue in *acnM* translational products; in aconitases in the tricarboxylic acid cycle this amino acid is isoleucine (indicated by boldface I). The references and accession numbers (if available), as well as contig numbers, are given in parentheses. Data for some of the organisms were obtained from the following websites: *B. cepacia*, *R. metallidurans* CH34, and *P. fluorescens*, <http://www.expasy.ch/cgi-bin/nicesite.pl?PS00161>; and *P. putida* KT2440, *P. syringae*, and *S. putrefaciens*, <http://www.tigr.org/cgi-bin/BlastSearch/blast.cgi?organism>.

product of ORF9 exhibited identity to a hypothetical gene product of *Rubrivivax gelatinosus*, probably a lipoprotein (accession no. T50918).

DISCUSSION

Although several pathways for propionic acid catabolism in bacteria have been described, it has been suggested that in *E. coli*, especially at a higher substrate concentration, the main route is α -oxidation of propionate via acrylyl-CoA and lactyl-CoA to pyruvate (56). However, this suggestion was based on some misleading enzyme activity measurements (50) and radiolabeling experiments (56). Nevertheless, results obtained in radiolabeling experiments did not allow the researchers to distinguish between α -oxidation and the 2-methylcitrate pathway, since the patterns of the labeled products formed were identical (i.e., decarboxylation of propionate at C-1). Therefore, the results obtained previously should be carefully reviewed. However, in the last few years there has been evidence that propionic acid is oxidized to pyruvate by the 2-methylcitrate pathway (MCC) in *E. coli*. In addition, activity of 2-methylcitric acid synthase, which is one of the key enzymes of the 2-methylcitrate pathway, has also been detected in *E. coli* (50), *S. enterica* (23), *R. eutropha* (8), *P. aeruginosa* (55), and *Pyrococcus furiosus* (16, 55). The other enzymes of this pathway, encoded by the *prpE* and *prpB* genes, were identified as a propionyl-CoA synthetase (PrpE) in *S. enterica* serovar Typhimurium LT2 (23) and a 2-methylisocitrate lyase (PrpB) in *E.*

coli and *Aspergillus nidulans* (10), respectively. Although it is evident that the MCC is probably used most often for catabolism of propionic acid in bacteria and that this pathway also occurs in some eukaryotes, the MCC has been studied in detail only in a few bacteria and in the eukaryote *A. nidulans* (9, 10) at a biochemical and molecular level. For most bacteria evidence for this pathway has been obtained only from genome sequence data.

This study revealed that the 2-methylcitric acid pathway is the predominant pathway for catabolism of propionic acid in *B. sacchari* IPT101^T. An understanding of this pathway and the availability of the genes of this pathway should allow workers to obtain improved strains of *B. sacchari* that are more suitable for fermentative production of poly(3HB-co-3HV) from sucrose and propionic acid.

In a previous study two classes of propionic acid-negative mutants of *B. sacchari* IPT101^T were obtained. Representatives of the first class might be affected in an unknown pathway, whereas representatives of the second class were defective in the MCC (41). Mutant IPT189, which belongs to the second class, exhibited the highest increase in the yield of 3HV units in the copolyester poly(3HB-co-3HV) from propionate. The results of growth experiments performed in a bioreactor, compared to the results of shaken flask experiments, revealed that the pathway affected in mutant IPT189 is important if low concentrations (0.02 to 0.04%, wt/vol) of propionic acid are present in the medium, whereas the other pathway of propi-

onic acid oxidation is more important if a higher concentration (0.1%, wt/vol) of propionic acid is present in the medium. More than 90% of the mutants obtained after enrichment, which was performed with high concentrations of this organic acid, were deficient in utilization of intermediates of the α -oxidation pathway (41).

Phenotypic complementation of such a mutant led to cloning of a 25.5-kbp *Hind*III fragment which carried a cluster of genes whose putative gene products exhibited strong similarities to the gene products of genes of the *prp* operon of *R. eutropha* and several other gram-negative bacteria (8). The gene products of this *prp* operon constitute the 2-methylcitric acid pathway in this mutant strain. In *B. sacchari* IPT101^T a putative aconitate hydratase gene (*acnM*) is also located downstream of *prpC*, as shown for the propionate cluster in several organisms. The *prpD* gene, which is probably involved in the isomerization reaction in the MCC of *S. enterica* serovar Typhimurium, as shown by the accumulation of 2-methylcitric acid (24) and in vitro conversion of 2-methylcitrate into 2-methylisocitrate (25), is missing at the *prp* locus of *B. sacchari*, as also observed for *R. metallidurans* CH34, *B. cepacia*, *Neisseria meningitidis* serogroups A and B, *Shewanella putrefaciens*, and *Pseudomonas syringae*. If the *acnM* and ORF5 gene products are also involved in conversion of 2-methylcitrate into 2-methylisocitrate, as in *R. eutropha* HF39, the *prpD* gene product is not required for a functional MCC in *B. sacchari* IPT101^T. If ORF5, ORF7, and ORF8 are also not required for a functionally active MCC, the *prp* locus of this strain consists of the minimal set of structural genes (*prpB*, *prpC*, *acnM*, and ORF5) which are required for the function of this cyclic pathway.

As shown by the reduced specific activities of 2-methylcitrate synthase and 2-methylisocitrate lyase in propionate-induced mutant IPT189 and by the complementation of mutant IPT189 by the *R. eutropha* HF39 *prpC* gene, the formation of 2-methylcitrate might influence the transcription of the *prp* locus in *B. sacchari* IPT101^T, as supposed for *S. enterica* serovar Typhimurium (33).

A number of authors have analyzed (by nuclear magnetic resonance) amino acids or other products formed in radiolabeling experiments to evaluate the relevance of propionate catabolism pathways. However, labeling tests allow simple distinctions between randomizing pathways (which lead to succinate) and nonrandomizing pathways (which lead to acetyl-CoA). The poly(3HB-co-3HV) production system could be used in a similar way. The poly(3HB-co-3HV) production system is a useful system for revealing the relative contributions of the various pathways to the catabolism of propionate since it generates large amounts of products, in contrast to the amounts of amino acids obtained in labeling studies, which make it difficult to analyze the labeled products. Independent of radiolabeling assays, the poly(3HB-co-3HV) production system can indicate the relevance of the different pathways if mutants are analyzed and if their $Y_{3HV/Prop}$ values are compared. Use of this system in previous studies (41) showed that in *B. sacchari* the 2-methylcitrate pathway operates better at low propionate concentrations (0.2 to 0.4 g · liter⁻¹; $Y_{3HV/Prop} = 1.2 \text{ g} \cdot \text{g}^{-1}$) than at higher propionate concentrations (1 g · liter⁻¹; $Y_{3HV/Prop} = 0.8 \text{ g} \cdot \text{g}^{-1}$); i.e., a mutation in the MCC resulted in more propionate available for 3HV synthesis and

therefore increased $Y_{3HV/Prop}$ in the presence of a low propionate concentration.

Moreover, the system provided evidence that a second minor pathway is present since the maximum theoretical yield (1.35 g · g⁻¹) was not achieved (41).

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