APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1996, p. 372–379 Vol. 62, No. 2
0099-2240/96/$04.00+0

Metabolic Pathway for Poly(3-Hydroxybutyrate-*co*-3-Hydroxyvalerate) Formation in *Nocardia corallina*: Inactivation of *mutB* by Chromosomal Integration of a Kanamycin Resistance Gene

HENRY E. VALENTIN* AND DOUGLAS DENNIS

Department of Biology, James Madison University, Harrisonburg, Virginia 22807

Received 3 August 1995/Accepted 7 November 1995

The gene encoding the large subunit of the methylmalonyl-coenzyme A (CoA) mutase in *Nocardia corallina* ($mutB_{Nc}$) was cloned. A 4.3-kbp *Bam*HI fragment containing almost the entire $mutB_{Nc}$ was identified by Southern hybridization experiments employing a digoxigenin-labeled probe deduced from *mutB* of *Streptomyces cinnamonensis*. $mutB_{Nc}$ was interrupted by insertion of a kanamycin resistance gene block (*mutB*::*kan* or *mutB*::*neo*) and introduced into *N. corallina* to obtain *mutB*-negative strains by homologous recombination. Four of sixteen kanamycin-resistant clones occurred via double-crossover events and harbored only the interrupted $mutB_{Nc}$. These exhibited no growth on odd-chain fatty acids in the presence of kanamycin but exhibited wild-type growth on even-chain fatty acids, glucose, and succinate. Whereas the wild type of *N. corallina* accumulates a copolyester of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) containing more than 60 mol% 3HV from most carbon sources, *mutB*-negative strains accumulated poly(3HB-*co*-3HV) containing only 2 to 6 mol% 3HV. Methylmalonyl-CoA mutase activity was not found in these clones. Therefore, this study provides strong evidence that the majority of 3HV units in poly(3HB-*co*-3HV) accumulated by *N. corallina* are synthesized via the methylmalonyl-CoA pathway.

Polyhydroxyalkanoates (PHAs) are bacterial storage compounds which are accumulated by a wide variety of different bacteria when a carbon source is available in excess, while growth is limited by the lack of an essential nutrient (1, 25). A large number of different hydroxy-fatty acids are incorporated into this polyester (27). However, most of these compounds are incorporated into PHA only if suitable carbon sources are provided as precursor substrates (27). Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [poly(3HB-*co*-3HV)] (2), and medium-chain-length PHAs (11, 28) are examples of PHAs other than poly(3HB) which are accumulated from simple carbohydrates. Among these, poly(3HB-*co*-3HV) is the only polyester which is produced on an industrial scale. Though several different organisms such as *Rhodococcus ruber*, *Nocardia lucida*, *Corynebacterium hydrocarboxydans* (2), and *Haloferax mediterranei* (20, 21) and members of some genera of the non-purple sulfur bacteria like *Rhodobacter*, *Rhodocyclus*, *Rhodopseudomonas*, and *Rhodospirillum* (13) are able to accumulate poly(3HB-*co*-3HV) from unrelated carbon sources, the metabolic pathways involved in the formation of this polyester are still not completely understood, and no molecular data are available yet. This is probably due to the fact that the most interesting polyesters of this type (highest 3HV levels) are accumulated by nocardiform bacteria and these organisms are largely inaccessible to molecular analysis.

When *Nocardia corallina* is incubated under nitrogen starvation and a carbon source is available in excess, it accumulates a copolyester consisting of 3HB and 3HV with 3HV as the predominant monomer (9). The amount of 3HV in poly(3HB-*co*-3HV) accumulated by this organism depends on the carbon source as well as the culture conditions and varies from 60 to 90 mol% on most substrates. A similar polyester is accumulated by *Rhodococcus ruber* (2). Nuclear magnetic resonance (NMR) spectroscopic studies in *Rhodococcus ruber* have suggested that the methylmalonyl-coenzyme A (CoA) pathway provides propionyl-CoA for the formation of 3HV units in poly(3HB-*co*-3HV) (31). However, the analysis of poly(3HB-*co*-3HV) accumulated from ^{13}C-labeled carbon sources did not reveal a definite pathway, and the biochemical data were not completely consistent with the NMR spectroscopic results. Because of the genetic and physiological similarity between *Rhodococcus ruber* and *N. corallina*, it is possible that 3HV units are synthesized via similar pathways in both organisms. Therefore, the *mutB* gene, encoding the large subunit of the methylmalonyl-CoA mutase, has been interrupted by chromosomal integration of a kanamycin resistance gene, to determine if it is the methylmalonyl-CoA pathway which provides 3HV units for the accumulation of poly(3HB-*co*-3HV) in *N. corallina.*

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. All strains and plasmids investigated in this study are listed in Table 1. *Escherichia coli* was grown at 37°C in Luria-Bertani (LB) medium (16) or on LB agar plates containing 16 g of agar per liter and the appropriate antibiotics at the indicated concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml. Precultures of *N. corallina* were grown in 50 ml of LB medium in 300-ml baffled Erlenmeyer flasks (225 rpm) at 30°C. For isolation of DNA, strains of *N. corallina* were grown in medium 148G (23). For analysis of PHA accumulation, the bacteria were grown for 48 h in an orbital shaker at 30°C in mineral salts medium (MSM) (22) containing 0.5 g of NH_4Cl per liter. To analyze PHA accumulation with valeric acid as carbon source, *N. corallina* strains were grown in 300-ml baffled Erlenmeyer flasks containing 50 ml of LB medium, harvested aseptically at the beginning of stationary-growth phase, washed once in nitrogen-free MSM, and resuspended in 50 ml of nitrogen-free MSM containing 0.25% (vol/vol) valeric acid. After 24 h of incubation at 30°C, an additional 0.25% (vol/vol) valeric acid was added. Cells were harvested after 48 h of incubation at 30°C.

To monitor colony growth, precultures of *N. corallina* were grown in LB

* Corresponding author. Mailing address: Department of Biology, James Madison University, Harrisonburg, VA 22807. Phone: (540) 568-3525. Fax: (540) 568-3333. Electronic mail address: Valenthe@vax1.acs.jmu.edu.

TABLE 1. Bacterial strains and plasmids[a]

Strain or plasmid	Relevant genotype	Description	Source or reference
Strains			
Agrobacterium tumefaciens		Wild type	Carolina Biologicals
Alcaligenes eutrophus H16		Wild type	DSM 428 (29)
Escherichia coli DH5α	*recA1 endA1* Δ(*lacZYA-argF*)		Gibco BRL
Nocardia corallina		Wild type, prototrophic	No. 724, Presque Isle Cultures, Presque Isle, Pa.
N. corallina 5	*mutB*$^+$ *mutB*::*kan*	Chromosomal insert of pJM9518 by single crossover	This study
N. corallina 7	*mutB*$^+$ *mutB*::*kan*	Multiple chromosomal insert of pJM9518	This study
N. corallina 10	*mutB*$^+$ *mutB*::*kan*	Chromosomal insert of pJM9518 by single crossover	This study
N. corallina 12	*mutB*::*kan*	Inactivation of *mutB* by insertion of *kan*	This study
N. corallina 13	*mutB*::*kan*	Inactivation of *mutB* by insertion of *kan*	This study
N. corallina A1	*mutB*::*neo*	Inactivation of *mutB* by insertion of *neo*	This study
Rhodospirillum rubrum		Wild type	No. 59, Presque Isle Cultures
Streptomyces cinnamonensis		Wild type	ATCC 15413
Plasmids			
pBluescript II SK$^-$	Ap^r *lacPOZ′*		Stratagene
pJM9516	*mutB*$^+$	pBluescript II SK$^-$ harboring fragment KK10 from *N. corallina*	This study
pJM9517	*mutB*$^+$	pBluescript II SK$^-$ harboring fragment BB4.3 from *N. corallina*	This study
pJM9518	*mutB*::*kan*	pBluescript II SK$^-$ harboring *mutB* from *N. corallina* inactivated by insertion of *kan*	This study
pJM9519	*mutB*::*neo*	pBluescript II SK$^-$ harboring *mutB* from *N. corallina* inactivated by insertion of *neo*	This study
pCY104	*cat kan tsr*	Shuttle vector for *Norcardia* spp. (Cm^r Km^r Ts^r) and *E. coli* (Cm^r Km^r)	31

[a] Abbreviations: Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; *kan*, kanamycin resistance gene from Tn*903*; Km^r, kanamycin resistance; *mutB*, gene encoding the large subunit of the methylmalonyl-CoA mutase of *N. corallina*; *neo*, kanamycin resistance gene from Tn*5*; *tsr*, thiostrepton resistance gene; Ts^r, thiostrepton resistance. *E. coli* genotypes are according to the work of Bachmann (4).

medium containing 200 μg of kanamycin if necessary and harvested at the beginning of stationary growth phase. After washing and resuspending of the bacteria in MSM, cells were streaked onto MSM agar plates containing 0.1% (vol/vol) fatty acids, 0.5% (wt/vol) sodium succinate, or 0.5% (wt/vol) glucose. Colony growth was monitored after 2 and 4 days of incubation at 30°C.

DNA isolation and manipulations. Isolation of total cellular DNA was done according to the method of Ausubel et al. (3). For isolation of total cellular DNA from strains of *N. corallina*, cells were grown in medium 158G (23). Cell lysis was performed as described previously (14). For standard plasmid isolation, DNA was purified by the alkaline-lysis method (16). Ligations were performed with T4 DNA ligase (Gibco BRL, Rockville, Md.) according to the manufacturer's specifications. Restriction digests and other molecular biology techniques were performed as described previously (16). Restriction endonucleases were obtained from Gibco BRL or New England Biolabs (Boston, Mass.). T4 DNA polymerase was obtained from Gibco BRL. DNA fragments were purified from agarose gels with the GeneClean Kit (Bio 101, La Jolla, Calif.) or Qiaquick Gel Extraction Kit (Qiagen Inc., Chatsworth, Calif.).

Electroporation. Plasmids were introduced into strains of *E. coli* by electroporation as described previously (19) with the Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.). For electroporation of *N. corallina* plasmid, DNA was purified from *E. coli* DH5α with the Qiagen Plasmid Kit (Qiagen Inc.). To obtain electrocompetent cells of *N. corallina*, a 250-ml culture in medium 148G (23) containing 1.5 mg of isonicotinic acid hydrazide per liter was inoculated with 2.5 ml of an overnight LB preculture. Cells were grown to an optical density of 0.3 (at 550 nm) and washed once with deionized water, and the cell pellet was resuspended in an equivalent volume of 35% (wt/vol) polyethylene glycol 1000. Competent cells were either used directly for electroporation or stored at −70°C. Electroporation of *N. corallina* was performed with Bio-Rad Gene Pulser cuvettes with a gap of 2 cm in 50- or 100-μl aliquots with settings of 2,500 V, 400 Ω, and 25 μF and 3 to 14 μg of plasmid DNA. After electroporation, the cells were immediately resuspended in 500 μl of R2L medium (15), transferred into a sterile 15-ml Falcon vial, mixed with 3 ml of R2L-agarose overlay medium (15), and plated onto predried S27M agar plates (15) containing 50 ml of agar medium. Plates were incubated for 16 h at 30°C. Subsequently, 1 ml of kanamycin stock solution was spread on the plates to obtain a final concentration of 200 μg of kanamycin per ml. Colonies appeared after 3 to 4 days of incubation at 30°C.

PCR. To amplify the *mutB*-specific DNA, total genomic DNA of *Streptomyces cinnamonensis* was isolated according to the method of Ausubel et al. (3) and an aliquot was incubated for 35 cycles with *mutB*-specific primers (upper primer, 5′-GAGTCCAACGCCTTCTACGC; lower primer, 5′-ACGAGCTTCGCCCAGAGCAG; purchased from Ransom Hill Bioscience, Inc., Ramona, Calif.) in a Thermolyne thermocycler (Temp•Tronic) with the GeneAmp kit (Perkin-Elmer Cetus, Norwalk, Conn.). Cycles included the following steps: 45 s of incubation at 94°C for denaturation, 1 min at 55°C for annealing, and 3 min at 72°C for extension. Temperature shifts were done at the highest possible rate.

To obtain purified template DNA for preparation of a *mutB*-specific DNA probe, the PCR products were separated on a 4% low-melting-point agarose gel and *mutB*-specific DNA was excised with a Pasteur pipette. The agar plug containing *mutB*-specific DNA was transferred into a microcentrifuge tube, 50 μl of H_2O was added, and the mixture was incubated for 10 min at 65°C. A digoxigenin (DIG)-labeled DNA probe was obtained by adding 5 μl of purified template DNA to a total volume of 100 μl containing 10 mM $MgCl_2$, 50 mM KCl, 10 μM each primer, 200 μM dCTP, 200 μM dATP, 200 μM dGTP, 150 μM dTTP, 50 μM DIG-11-dUTP (Boehringer Mannheim, Indianapolis, Ind.), and 2.5 U of *Taq* DNA polymerase in 10 mM Tris-HCl, pH 8.3. The reaction mixture was incubated for 35 cycles in a thermocycler as described above. The DIG-labeled probe was separated from DIG-11-dUTP monomers by ethanol precipitation. Approximately 25 ng of DIG-labeled DNA probe was sufficient for 10 ml of hybridization buffer.

Southern hybridization experiments. DNA samples were separated on 0.8% (wt/vol) agarose gels in Tris-acetate-EDTA buffer according to the method of Maniatis et al. (16). The DNA was transferred onto MagnaGraph nylon membranes (Micron Separations, Inc., Westboro, Mass.) with a PosiBlot apparatus (Stratagene, La Jolla, Calif.) according to the manufacturer's protocol.

Colony hybridization was done according to a modified method of Grunstein and Hogness (10). *E. coli* clones were transferred with toothpicks onto MagnaGraph nylon membranes (Micron Separations, Inc.) placed on LB-ampicillin medium and onto replica plates of the same medium. The plates were incubated for 8 h at 37°C. Replica plates were stored at 4°C. For amplification of plasmids, the nylon membrane was transferred onto an LB plate containing 10 μg of chloramphenicol per ml and incubated for 12 h at 37°C. All agar media used for colony hybridization experiments were poured in Bioassay dishes (Nunc A/S, Kamstrupvej, Denmark). Cell lysis was performed by placing the membrane for 3 min onto a 10% (wt/vol) sodium dodecyl sulfate (SDS) solution. Subsequently, the membranes were placed for 5 min each on 1.5 M NaCl in 0.5 M NaOH, on 1.5 M NaCl in 0.5 M Tris-HCl (pH 7.6), and on 2× SSC (1× SSC is 0.15 M NaCl

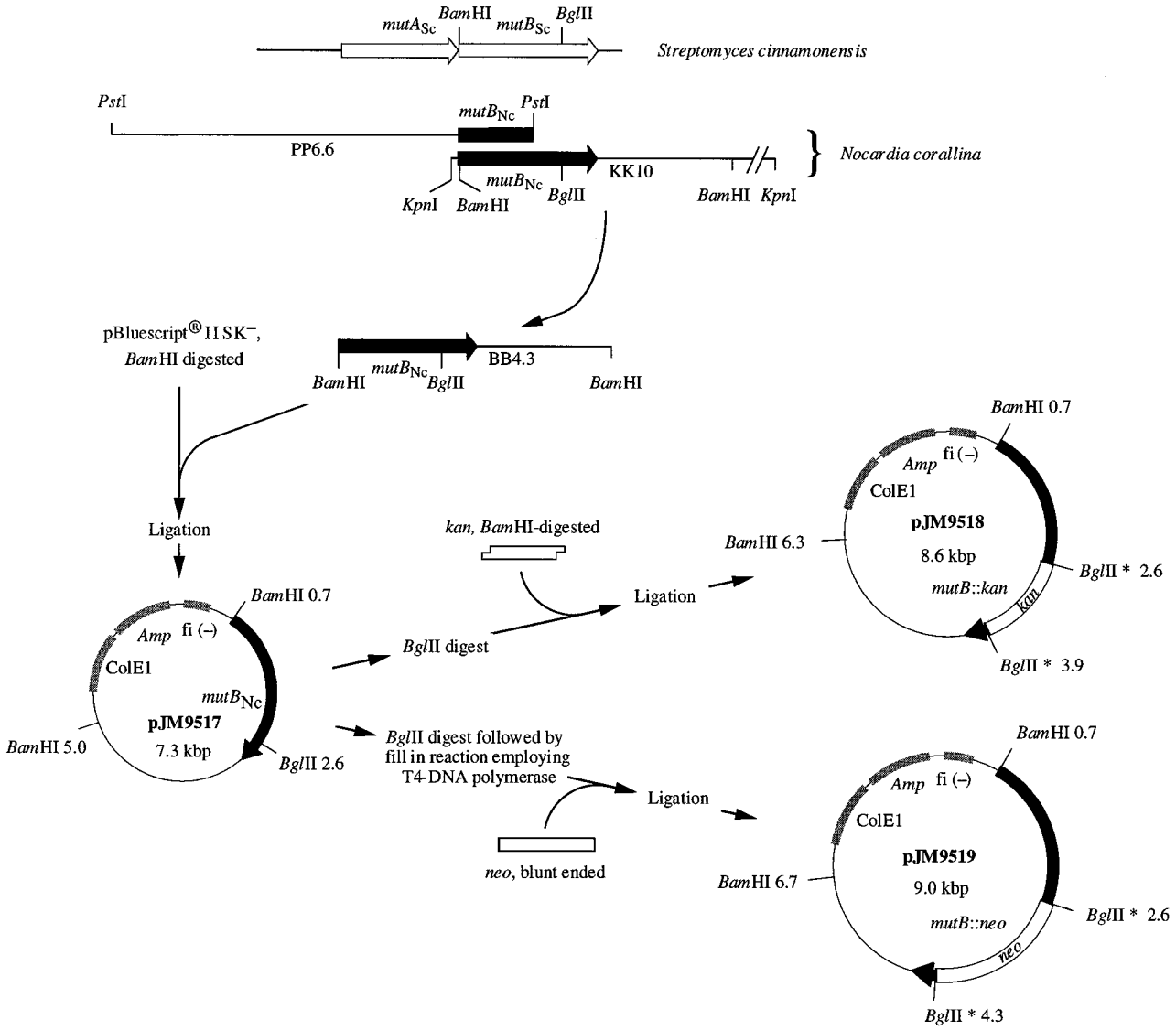

FIG. 1. Construction of hybrid plasmids used for chromosomal integration. Fragments PP6.6 and KK10 were cloned from chromosomal DNA of *N. corallina*. Restriction sites labeled by an asterisk have been destroyed by the cloning procedure. Abbreviations: *Amp*, ampicillin resistance; ColE1, origin of replication; fi (−), origin of replication of the filamentous phage f1; *kan*, kanamycin resistance of Tn*903* (Pharmacia, La Jolla, Calif.); $mutB_{Nc}$, *mutB* gene encoding the large subunit of the methylmalonyl-CoA mutase of *N. corallina*; *mutB*::*kan* and *mutB*::*neo*, *mutB* gene encoding the large subunit of the methylmalonyl-CoA mutase of *N. corallina*, interrupted at the *Bgl*II site by the insertion of a kanamycin resistance gene; $mutA_{Sc}$ and $mutB_{Sc}$, genes encoding the small and the large subunit of the methylmalonyl-CoA mutase of *S. cinnamonensis*, respectively; *neo*, kanamycin resistance from Tn*5*.

plus 0.015 M sodium citrate). Cell debris was removed with a spatula, while the membranes were kept wet on 2× SSC. Finally, DNA was fixed by UV cross-linking in a UV Stratalinker 1800 (Stratagene) according to the manufacturer's protocol, and membranes were baked for 2 h at 80°C.

Hybridization experiments were performed at 68°C in an Autoblot hybridization oven (Bellco Biotechnology, Vineland, N.J.) with ExpressHyb hybridization solution (Clontech Laboratories, Palo Alto, Calif.) according to the supplier's protocol. The membranes were washed with 0.1% SDS in 2× SSC at 60°C. Blots were developed with the DIG DNA Labeling and Detection Kit (Boehringer Mannheim) according to the manufacturer's protocol.

Recombinant plasmids. Plasmids pJM9518 and pJM9519 were obtained by cloning the kanamycin resistance gene of Tn*903* (*kan*; Pharmacia Biotech, Inc., Piscataway, N.J.) or of Tn*5* (*neo*), respectively, into a single *Bgl*II site which was located in the *mutB* gene of *N. corallina* ($mutB_{Nc}$) (Fig. 1).

DNA sequence analysis. DNA sequences were analyzed by PCR with a SequiTherm Long-Read cycle sequencing kit LC (Epicentre Technologies) and an automated DNA sequencer, model 4000 (Li-COR, Inc., Lincoln, Nebr.), according to the manufacturer's protocols.

Quantitative and qualitative analysis of PHA polyesters. To determine the polyester content and composition, approximately 5 to 14 mg of dry lyophilized cells was subjected to methanolysis in the presence of methanol-sulfuric acid (85:15 [vol/vol]) according to the method of Braunegg et al. (7) as modified by Brandl et al. (6). Methylesters were separated as described by Slater et al. (24). Poly(3HB-*co*-3HV) containing 14% (wt/wt) 3HV (Aldrich, Milwaukee, Wis.) was used for calibration.

Enzymatic analysis. To monitor methylmalonyl-CoA mutase activity, strains of *N. corallina* from the early-stationary-growth phase grown in 250 ml of medium 148G (23) were harvested aseptically, washed once in MSM, and resuspended in an equal amount of MSM containing 1% (wt/vol) glycine and 0.25% (vol/vol) valeric acid. For induction of the methylmalonyl-CoA mutase, the cultures were incubated for 16 h at 30°C while being shaken at 225 rpm. Subsequently, cells were harvested and washed once in 50 mM potassium phosphate (pH 7.4) containing 5 mM EDTA and 10% (wt/vol) glycerol (MCM buffer), and the cell pellet was stored at −70°C. For cell disruption, the samples were thawed on ice and sonicated in 5-ml aliquots with a Fisher 300 sonicator (tip diameter, 19 mm) at 90% of the maximum setting. Sonication consisted of five cycles of 10-s bursts followed by 20-s breaks. During sonication, the samples were cooled on ice. Cell debris and nondisrupted cells were removed by 15 min of centrifugation at 25,000 × *g*. Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad Laboratories).

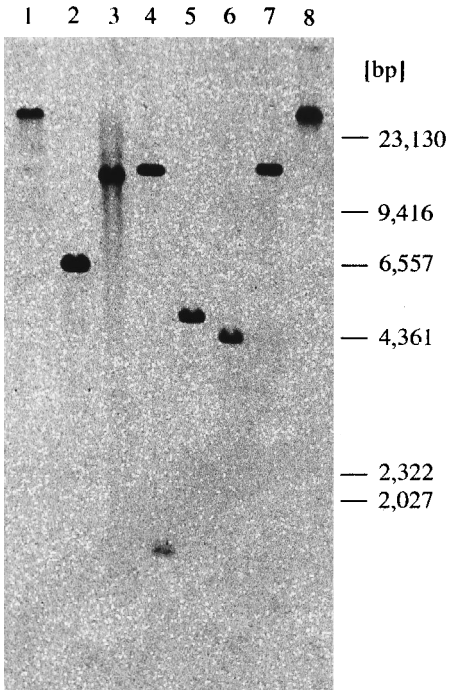

FIG. 2. Probing for genes encoding the large subunit of the methylmalonyl-CoA mutase. Total genomic DNA from different sources was analyzed in a Southern hybridization experiment with a *mutB*-specific DNA probe. Lanes 1 to 4 contain *Eco*RI-digested total genomic DNA of *Alcaligenes eutrophus*, *Agrobacterium tumefaciens*, *S. cinnamonensis*, and *Rhodospirillum rubrum*, respectively. Lanes 5 to 8 contain *Apa*I-, *Bam*HI-, *Cla*I-, and *Eco*RI-digested total genomic DNA of *N. corallina*, respectively.

The methylmalonyl-CoA mutase assay was done by a modified method of Birch et al. (5). A volume of 2 μl of 1 mM coenzyme B12 solution in H_2O was added to 200 μl of MCM containing 0.625 mg of protein per ml, and the solution was incubated for 10 min at 30°C in the dark. The reaction was started by adding 50 μl of 10 mM methylmalonyl-CoA. After 5 to 30 min of incubation at 30°C, the reaction was stopped by heat inactivation at 100°C for 10 min. Subsequently, the samples were lyophilized and subjected to methanolysis. Methylesters were analyzed quantitatively as described above. The disodium salt of succinic acid and methylmalonic acid subjected to methanolysis were used for calibration. One unit of methylmalonyl-CoA mutase activity was defined as 1 μmol of succinate formed · min^{-1}.

Chemicals. All chemicals were reagent grade and came from Sigma Chemical Company unless otherwise noted.

RESULTS

Cloning of *mutB* from *N. corallina*. Since the DNA sequences of several methylmalonyl-CoA mutases have been published recently (5, 11, 18, 30), we were able to deduce a specific probe to screen a *N. corallina* genomic library for genes encoding the methylmalonyl-CoA mutase. The deduced amino acid sequences of the methylmalonyl-CoA mutases from humans (accession numbers M65131, M2290, and M65022 [11]), mice (accession number X51941 [30]), *Propionibacterium shermanii* (accession number X14965 [18]), *Phosphyromonas gingivalis* (accession number L30136), and *S. cinnamonensis* (accession number L10064 [5]) were compared to identify conserved regions. All known prokaryotic methylmalonyl-CoA mutases were encoded by two open reading frames (*mutA* and *mutB*). Among these, the large subunits of the methylmalonyl-CoA mutases encoded by *mutB* exhibited the highest similarity. The deduced amino acid sequences of bacterial *mutB* genes were more than 57% identical, while the identity to eucaryotic genes was more than 50%. A pair of primers was chosen to amplify a highly conserved region of 648 bp within *mutB* of *S. cinnamonensis* occurring from nucleotides 3,464 to 4,111 (5). The amplification product was used to prepare a *mutB*-specific DNA probe as described above. Hybridization experiments employing total genomic DNA of *Alcaligenes eutrophus* H16, *Agrobacterium tumefaciens*, *S. cinnamonensis*, *Rhodospirillum rubrum*, *N. corallina* (Fig. 2), and *E. coli* (data not shown) revealed specific single signals, suggesting a high specificity of the probe. Only *Eco*RI-digested total genomic DNA of *Rhodospirillum rubrum* exhibited a second signal at a fragment size of 1.6 kbp (Fig. 2), which might be the result of an *Eco*RI site within the homologous sequence of the probe.

The *mutB* gene from *N. corallina* ($mutB_{Nc}$) was identified in a genomic library of *Kpn*I-digested DNA and in a genomic library of *Pst*I-digested DNA by colony hybridization, revealing fragments of 10 kbp (KK10) and 6.6 kbp (PP6.6), respectively (Fig. 1). Further hybridization experiments led to the identification of a 4.3-kbp *Bam*HI fragment (BB4.3; Fig. 1) harboring $mutB_{Nc}$. Partial DNA sequence analysis revealed that all but 45 nucleotides at the 5′ end of the $mutB_{Nc}$ open reading frame were encoded on fragment BB4.3 (Fig. 1).

Isolation of $mutB_{Nc}$-negative mutants. To obtain $mutB_{Nc}$-negative mutants, the 4.3-kbp *Bam*HI fragment harboring $mutB_{Nc}$ was cloned in pBluescript II SK^- (pJM9517). Cleavage with several restriction enzymes and partial DNA sequence

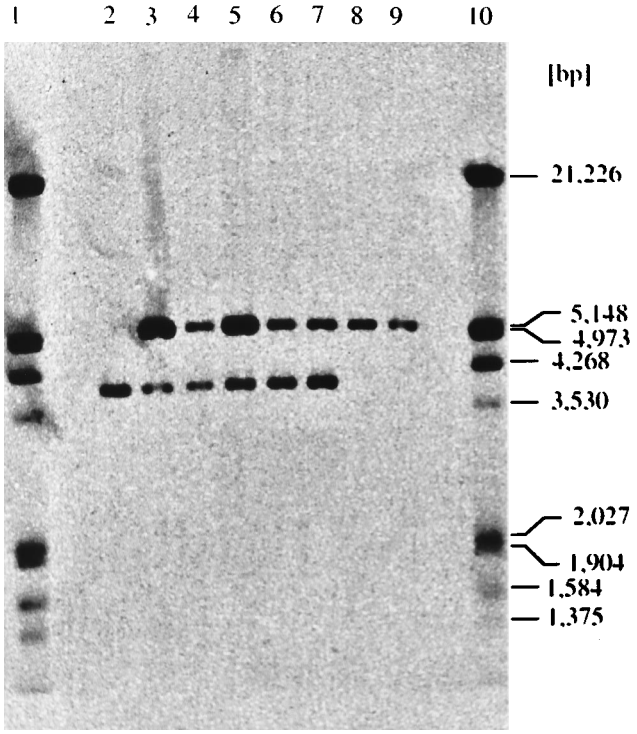

FIG. 3. Screening of kanamycin-resistant *N. corallina* clones for double-crossover events. *Bam*HI-digested total genomic DNA of kanamycin-resistant *N. corallina* clones was separated in an 0.8% (wt/vol) Tris-acetate gel, transferred onto a nylon membrane, and analyzed in a Southern hybridization experiment with a *mutB*-specific probe. Lanes 1 and 10 contain DIG-labeled molecular weight markers (Boehringer Mannheim). Lane 2 contains total genomic DNA of the *N. corallina* wild type, and lanes 3 to 9 contain total genomic DNA of *N. corallina* 1, 5, 7, 10, 11, 12, and 13, respectively. The signal running at the lower molecular weight represents the wild-type fragment, whereas the signal at the higher molecular weight represents *mutB* harboring the kanamycin resistance gene. The shift of the wild-type signal to a lower molecular weight compared with Fig. 2 is probably a result of an increased molecular weight of the DIG-labeled DNA molecular weight marker.

TABLE 2. Colony growth of representative *N. corallina* strains

Strain of *N. corallina*	Genotype	Growth on carbon source[a]											
		Acetate		Propionate		Valerate		Hexanoate		Succinate		Glucose	
		2 d	4 d	2 d	4 d	2 d	4 d	2 d	4 d	2 d	4 d	2 d	4 d
Wild type	*mutB*$^+$	++	++	++	++	++	++	+	+	++	++	+++	+++
5	*mutB*$^+$ *mutB*::*kan*	++	++	++	++	++	++	+	+	++	++	+++	+++
7	*mutB*$^+$ *mutB*::*kan*, multiple insertions	++	++	++	++	++	++	+	+	++	++	+++	+++
10	*mutB*$^+$ *mutB*::*kan*	++	++	++*	++*	++*	++*	+	+	++	++	+++	+++
12	*mutB*::*kan*	++	++	−	−	−	−	+	+	++	++	+++	+++
13	*mutB*::*kan*	++	++	−	−	−	−	+	+	++	++	+++	+++
A1	*mutB*::*neo*	++	++	−	−	−	−	+	+	++	++	+++	+++

[a] Colony growth was monitored on MSM (22) containing 0.1% (vol/vol) fatty acids or 0.5% (wt/vol) succinate, disodium salt, or glucose. Abbreviations: d, days (incubation period); − to +++, no colony growth to very good growth; *, uneven colony growth (fewer colonies than the wild type and different colony size).

analysis revealed a unique *Bgl*II site which was found in *mutB* of *S. cinnamonensis* at the same location (5) (Fig. 1). Therefore, the kanamycin resistance gene of either Tn*903* (*kan*) or Tn*5* (*neo*) was cloned into this *Bgl*II site (Fig. 1), resulting in the separation of approximately 540 nucleotides belonging to the 3′ end of $mutB_{Nc}$ from the main part of the gene (Fig. 1). Plasmids pJM9518 and pJM9519 were introduced into *N. corallina* by electroporation. Transformation efficiencies under the described conditions were 10^5 to 10^6/μg of DNA with the shuttle vector pCY104 (32). During a series of nine electroporation experiments using either 7.4 or 13.3 μg of pJM9519, seven kanamycin-resistant clones were obtained. Two of these clones were found to be spontaneous kanamycin-resistant mutants. The efficiency of integration was one integration event per 15 μg of plasmid DNA. The source of the kanamycin resistance cassette (*kan* or *neo*) as well as its orientation exhibited no significant effect on the experiments. Subsequently, kanamycin-resistant clones were screened to see whether they were spontaneous mutants or derived via single- or double-crossover recombinational events. *Bam*HI-digested total genomic DNA of all mutants was analyzed by Southern hybridization experiments, resulting in the identification of four different genotypes. The first genotype exhibited the wild-type signal of $mutB_{Nc}$ at 4.3 kbp in fragment size and a second signal exhibiting the same intensity as the first signal at 5.5 kbp (6.0 kbp if pJM9519 was used [Fig. 3, lanes 4, 6, and 7]). The second signal resulted from the integration of $mutB_{Nc}$ carrying the kanamycin resistance gene ($mutB_{Nc}$::*kan* or $mutB_{Nc}$::*neo*). The second genotype exhibited both of these signals, but the latter signal was significantly stronger than the signal of the intact $mutB_{Nc}$, indicating multiple insertions (Fig. 3, lanes 3 and 5). The third genotype exhibited only one signal at 5.5 kbp (6.0 kbp if pJM9519 was used [Fig. 3, lanes 8 and 9]), indicating that the intact wild-type $mutB_{Nc}$ was not present in these clones. The fourth genotype, which was found in two mutants, exhibited signals at fragment sizes of either more than 6 kbp or less than 4.3 kbp in addition to the wild-type $mutB_{Nc}$ signal (data not shown).

As a result of Southern hybridization experiments, 4 of 16 kanamycin-resistant clones were found to be *mutB* negative. One of these clones was obtained by chromosomal integration of the Tn*5* kanamycin resistance gene, and three *mutB*-negative clones contained the kanamycin resistance gene of Tn*903* as a chromosomal integrant.

Utilization of carbon sources and accumulation of poly(3-hydroxyalkanoic acids). Growth characteristics of kanamycin-resistant mutants were analyzed on mineral agar salts (MSM) plates (22) containing 0.1% (vol/vol) sodium salt of fatty acids or 0.5% (wt/vol) sodium succinate or glucose. All kanamycin-resistant strains exhibited wild-type growth on even-chain fatty acids, succinate, and glucose. Most mutants derived from a single-crossover event and all mutants with multiple insertions of $mutB_{Nc}$::*kan* in addition to the intact $mutB_{Nc}$ gene exhibited no difference in growth on fatty acids, succinate, or glucose

TABLE 3. PHA accumulation and enzyme activity of representative *N. corallina* strains

Strain of *N. corallina*	Genotype	Activity of methylmalonyl-CoA mutase (U/g of protein)	Content and composition with carbon source[a]								
			Glucose			Succinate			Valerate		
			PHA content (% of CDW)	PHA composition (mol%)		PHA content (% of CDW)	PHA composition (mol%)		PHA content (% of CDW)	PHA composition (mol%)	
				3HB	3HV		3HB	3HV		3HB	3HV
Wild type	*mutB*$^+$	9.3	2.4	39.7	60.3	0.4	30.1	69.9	5.6	2.7	97.3
5	*mutB*$^+$ *mutB*::*kan*	28.0	3.3	40.5	59.5	0.7	15.4	84.6	7.9	<0.5	>99.5
7	*mutB*$^+$ *mutB*::*kan*, multiple insertions	28.5	2.1	49.4	50.6	1.2	37.4	62.6	17.2	0.6	99.4
10	*mutB*$^+$ *mutB*::*kan*	15.5	3.1	97.7	2.3	0.9	94.8	5.2	5.5	7.6	92.4
12	*mutB*::*kan*	>0.1	2.9	98.1	1.9	1.0	95.7	4.3	4.9	1.8	98.2
13	*mutB*::*kan*	>0.1	2.6	97.6	2.4	1.3	96.1	3.9	8.4	1.2	98.8
A1	*mutB*::*neo*	>0.1	0.9	96.0	4.0	1.0	96.8	3.2	1.9	<0.5	>99.5

[a] For analyses of PHA accumulation, the organisms were grown on MSM (22) containing 0.5 g of NH_4Cl per liter and 0.5% (wt/vol) glucose or sodium succinate. To analyze PHA accumulation on valeric acid, the strains were grown in a two-step culture. Abbreviations: CDW, cellular dry weight; 3HB, 3-hydroxybutyric acid; 3HV, 3-hydroxyvaleric acid.

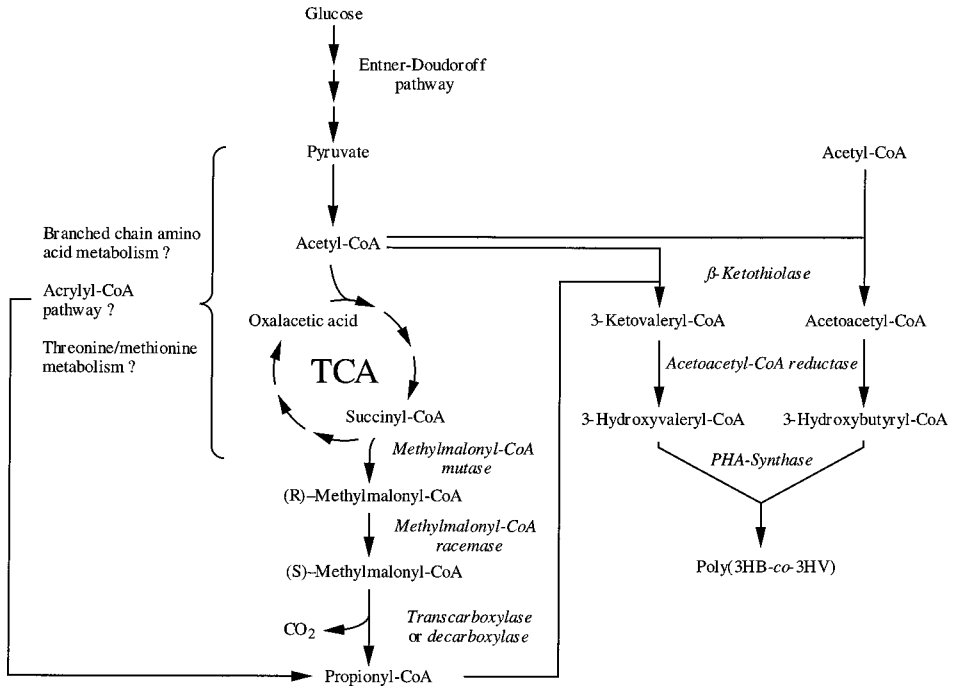

FIG. 4. Putative metabolic pathways involved in poly(3HB-*co*-3HV) formation in *N. corallina*. The majority of 3HV is synthesized via the methylmalonyl-CoA pathway. However, approximately 5 mol% 3HV seems to be synthesized via a second metabolic pathway, which might involve the branched chain amino acid metabolism, the acrylyl-CoA pathway, or the threonine/methionine metabolism. TCA, tricarboxylic acid cycle.

compared with the wild type (Table 2). In contrast, *mutB*-negative strains did not grow on odd-chain fatty acids but exhibited wild-type growth on even-chain fatty acids, succinate, and glucose. Some mutants which were obtained by a single-crossover event, such as *N. corallina* 10, exhibited a longer log phase than the wild type when grown on odd-chain fatty acids and exhibited different colony sizes, indicating a low stability of the mutation. However, these strains did not grow with odd-chain fatty acids as carbon and energy source in liquid MSM if antibiotics were present.

Accumulation of PHA was investigated by growing the strains in 50 ml of MSM containing 0.5 g of NH_4Cl per liter in 300-ml baffled Erlenmeyer flasks. Cultures were inoculated by adding 1 ml from an LB preculture. All strains investigated in this study accumulated similar amounts of PHA on glucose and succinate as carbon source (Table 3). However, the composition of the polyester exhibited dramatic changes in those strains which exhibited changes in their growth characteristics on odd-chain fatty acids compared with the wild type (Tables 2 and 3). While the wild type and those strains which grew like the wild type on odd-chain fatty acids accumulated a polyester mainly consisting of 3HV, those mutants which lost their ability to utilize odd-chain fatty acids for growth accumulated PHA containing only 1.9 to 5.2 mol% 3HV (Table 3). A similar polyester was accumulated by mutants such as *N. corallina* 10 (Table 3). However, if PHA accumulation was analyzed in a two-step accumulation experiment and sodium valerate was supplied as carbon and energy source, all mutants accumulated a polyester containing more than 90 mol% 3HV as it was found in the wild type.

Activity of methylmalonyl-CoA mutase. While the *N. corallina* wild type and mutants derived from single-crossover events exhibited significant methylmalonyl-CoA mutase activity after 12 to 16 h of incubation with valeric acid as carbon source, no methylmalonyl-CoA mutase activity was found in *mutB*-negative strains (Table 3). Interestingly, *N. corallina* 10, which accumulated PHA of similar composition as that found in the *mutB*-negative strains but which grew on odd-chain fatty acids, clearly exhibited an active methylmalonyl-CoA mutase (Tables 2 and 3).

DISCUSSION

This paper investigates the function of the methylmalonyl-CoA mutase in poly(3HB-*co*-3HV) formation by *N. corallina*. Mutants deficient in *mutB*, which encodes the large subunit of the methylmalonyl-CoA mutase, exhibited no methylmalonyl-CoA mutase activity and accumulated a polyester which differed significantly in its composition from PHA accumulated by the wild type. The molar fraction of 3HV decreased from approximately 60 mol% to less than 5 mol% when glucose was provided as carbon and energy source. When valeric acid was supplied as carbon source for PHA accumulation, the predominant monomer in PHA was 3HV in all strains. This clearly indicates the importance of the methylmalonyl-CoA mutase for the formation of 3HV monomers in *N. corallina* and complements the NMR spectroscopic data of Williams et al. (31), which indicated a similar pathway for 3HV formation in *Rhodococcus ruber*. However, all *mutB*-negative strains still accumulated poly(3HB-*co*-3HV) containing 2 to 5 mol% 3HV, suggesting that a second metabolic pathway for the formation of 3HV units is present. Certain disharmonies in the labeling pattern of poly(3HB-*co*-3HV) accumulated from ^{13}C-labeled succinic acid by *Rhodococcus ruber* led Williams et al. (31) to a similar proposal. A comparable molar fraction of 3HV units has been found in a mutant of *Alcaligenes eutrophus* which is thought to provide propionyl-CoA for the formation of 3HV units via the branched chain amino acid metabolic pathway (26). Therefore, this pathway may also be active in *N. corallina* (Fig. 4). Alternatively, propionyl-CoA could also be synthe-

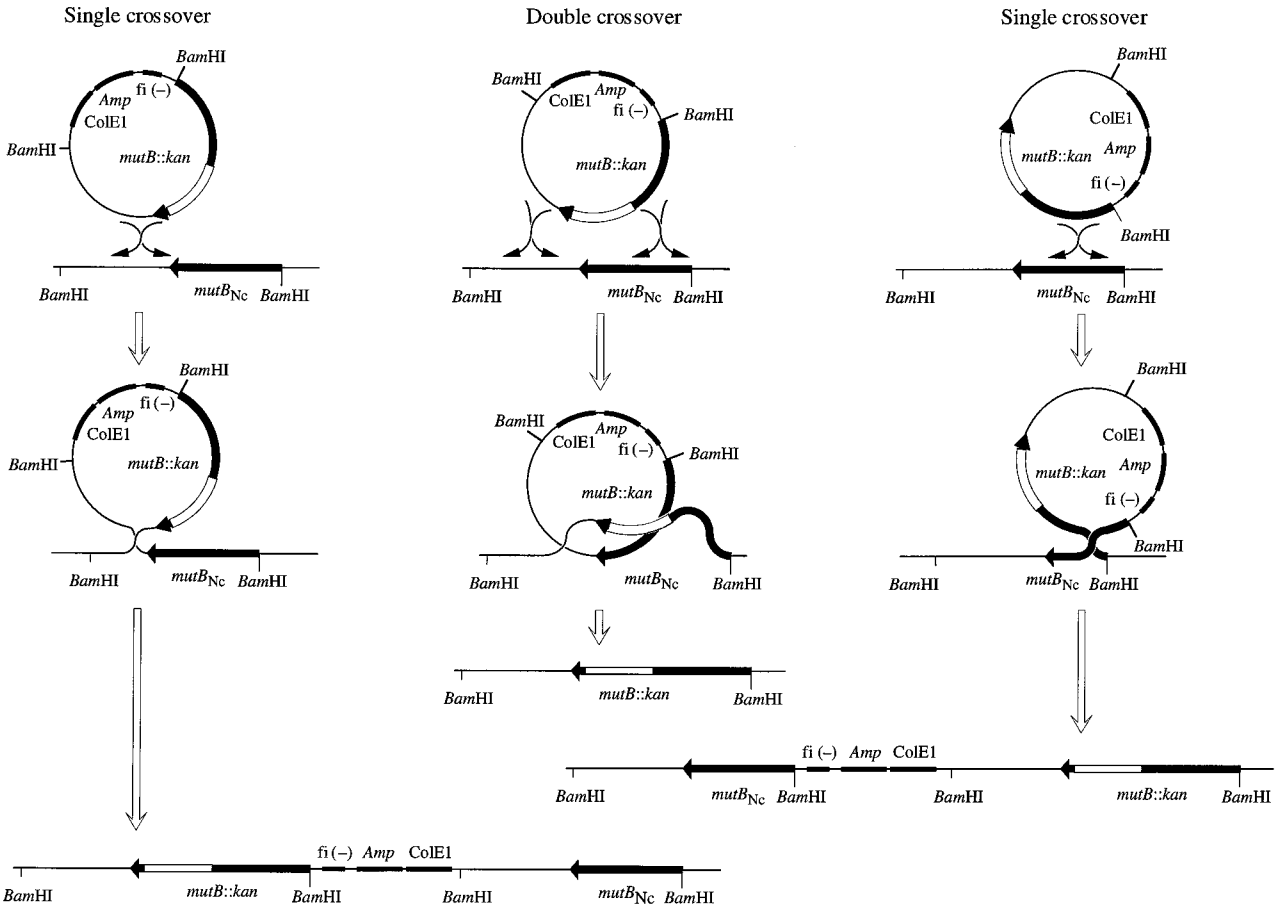

FIG. 5. Chromosomal integration of the *mutB*$_{Nc}$::*kan* gene by single and double crossover. Depending on the location of a single crossover, it can cause different polar effects as a result of upstream or downstream integration of the plasmid. For abbreviations, see the legend to Fig. 1.

sized via the acrylyl-CoA pathway (8) or via the threonine/methionine metabolism (17).

The elimination of one pathway may facilitate further studies which aim to identify the second metabolic pathway. NMR spectroscopic studies employing ^{13}C-labeled carbon sources might exhibit less complicated and more easily interpretable spectra than previous experiments (31). However, the formation of methylmalonyl-CoA by low background activities of the methylmalonyl-CoA mutase cannot be completely excluded on the basis of the present data.

Interestingly, one mutant, *N. corallina* 10, harboring the wild-type *mutB* as well as the interrupted *mutB* (most probably as a result of a single-crossover event), accumulated a polyester of similar composition as that in *mutB*-negative strains. This may be the result of polar effects. Depending on the location of the crossover, the mutagenized *mutB* may be located upstream or downstream of the wild-type *mutB* (Fig. 5) and, therefore, separate the wild-type gene from its promoter or a second open reading frame such as the gene encoding the methylmalonyl-CoA racemase or methylmalonyl-CoA decarboxylase (transcarboxylase). However, these mutants differed in their growth characteristics on odd-chain fatty acids from *mutB*-negative strains. In contrast to *mutB*-negative strains, they were able to utilize odd-chain fatty acids as carbon and energy source for colony growth and methylmalonyl-CoA mutase activity was found in crude extracts. The observed differences in colony sizes may indicate spontaneous revertants and, therefore, an instability of this mutation.

ACKNOWLEDGMENTS

We thank Cassie Paup for technical assistance and Dana Kolibachuk and Ken Gonyer for critical evaluation of the manuscript. We also thank J. Chiao for providing plasmid pCY104 and Claudia Grzeszik as well as Ivor Knight for valuable hints.

This work was supported by National Science Foundation grant MCB-9120428.

REFERENCES

1. **Anderson, A. J., and E. A. Dawes.** 1990. Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiol. Rev. **54:**450–472.
2. **Anderson, A. J., G. W. Haywood, D. R. Williams, and E. A. Dawes.** 1990. The production of polyhydroxyalkanoates from unrelated carbon sources, p. 119–129. *In* E. A. Dawes (ed.), Novel biodegradable microbial polymers. Kluwer Academic Publishers, Dordrecht, The Netherlands.
3. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1987. Current protocols in molecular biology. Greene Publishing Associates and John Wiley & Sons, New York.
4. **Bachmann, B. J.** 1987. Linkage map of *Escherichia coli* K-12, edition 7, p. 807–876. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 2. American Society for Microbiology, Washington, D.C.
5. **Birch, A., A. Leiser, and J. A. Robinson.** 1993. Cloning, sequencing, and expression of the gene encoding methylmalonyl-coenzyme A mutase from

Streptomyces cinnamonensis. J. Bacteriol. **175:**3511–3519.
6. **Brandl, H., R. A. Gross, R. W. Lenz, and R. C. Fuller.** 1988. *Pseudomonas oleovorans* as a source of poly(β-hydroxyalkanoates) for potential applications as biodegradable polyesters. Appl. Environ. Microbiol. **54:**1977–1982.
7. **Braunegg, G., B. Sonnleitner, and R. B. Lafferty.** 1978. A rapid gas chromatographic method for the determination of poly-β-hydroxybutyric acid in microbial biomass. J. Appl. Microbiol. **6:**29–37.
8. **Cardon, B. P., and H. A. Barker.** 1947. Amino acid fermentations by *Clostridium propionicum* and *Diplococcus glycinophilus*. Arch. Biochem. **12:**165–180.
9. **Dennis, D., H. G. Rhie, and J. Stauffer.** 1995. Unpublished results.
10. **Grunstein, M., and D. Hogness.** 1975. Colony hybridization: a method for the isolation of cloned DNA's that contain a specific gene. Proc. Natl. Acad. Sci. USA **72:**3961–3965.
11. **Haywood, G. W., A. J. Anderson, D. F. Ewing, and W. A. Dawes.** 1990. Accumulation of a polyhydroxyalkanoate containing primarily 3-hydroxydecanoate from simple carbohydrate substrates by *Pseudomonas* sp. strain NCIMB40135. Appl. Environ. Microbiol. **56:**3354–3359.
12. **Jansen, R., F. Kalousek, W. A. Fenton, L. E. Rosenberg, and F. D. Ledley.** 1989. Cloning of full-length methylmalonyl-CoA mutase from a cDNA library using the polymerase chain reaction. Genomics **4:**198–205.
13. **Liebergesell, M., E. Hustede, A. Timm, A. Steinbüchel, R. C. Fuller, R. W. Lenz, and H. G. Schlegel.** 1991. Formation of poly(3-hydroxyalkanoates) by phototrophic and chemolithotrophic bacteria. Arch. Microbiol. **155:**415–421.
14. **Loeffelholz, M. J., and D. R. Scholl.** 1989. Method for improved extraction of DNA from *Nocardia asteroides*. J. Clin. Microbiol. **27:**1880–1881.
15. **Madon, J., and R. Hütter.** 1991. Transformation system for *Amycolatopsis* (*Nocardia*) *mediterranei*: direct transformation of mycelium with plasmid DNA. J. Bacteriol. **173:**6325–6331.
16. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. **Marcus, J. P., and E. E. Dekker.** 1993. Threonine formation via the coupled activity of 2-amino-3-ketobutyrate coenzyme A lyase and threonine dehydrogenase. J. Bacteriol. **175:**6505–6511.
18. **Marsh, E. N., N. McKie, N. K. Davis, and P. F. Leadlay.** 1989. Cloning and structural characterization of the genes coding for adenosylcobalamin-dependent methylmalonyl-CoA mutase from *Propionibacterium shermanii*. Biochem. J. **260:**345–352.
19. **Miller, J. F.** 1988. Bacterial electroporation. Mol. Biol. **5:**1–4.
20. **Rodriguez-Valera, F., and J. A. G. Lillo.** 1990. Halobacteria as producers of poly-β-hydroxyalkanoates, p. 425–426. *In* E. A. Dawes (ed.), Novel biodegradable microbial polymers. Kluwer Academic Publishers, Dordrecht, The Netherlands.
21. **Rodriguez-Valera, F., and J. A. G. Lillo.** 1992. Halobacteria as producers of poly-β-hydroxyalkanoates. FEMS Microbiol. Rev. **103:**181–186.
22. **Schlegel, H. G., H. Kaltwasser, and G. Gottschalk.** 1961. Ein Submersverfahren zur Kultur wasserstoffoxydierender Bakterien: Wachstumsphysiologische Untersuchungen. Arch. Mikrobiol. **38:**209–222.
23. **Schupp, T., and M. Divers.** 1986. Protoplast preparation and regeneration in *Nocardia mediterranei*. FEMS Microbiol. Lett. **36:**159–162.
24. **Slater, S., T. Gallaher, and D. Dennis.** 1992. Production of poly-(3-hydroxybutyrate-co-3-hydroxyvalerate) in a recombinant *Escherichia coli* strain. Appl. Environ. Microbiol. **58:**1089–1094.
25. **Steinbüchel, A.** 1991. Polyhydroxyalkanoic acids, p. 123–213. *In* D. Byrom (ed.), Biomaterials. Macmillan Publishers Ltd., Basingstoke, United Kingdom.
26. **Steinbüchel, A., and U. Pieper.** 1992. Production of a copolyester of 3-hydroxybutyric acid and 3-hydroxyvaleric acid from single unrelated carbon sources by a mutant of *Alcaligenes eutrophus*. Appl. Microbiol. Biotechnol. **37:**1–6.
27. **Steinbüchel, A., and H. E. Valentin.** 1995. Diversity of bacterial polyhydroxyalkanoic acids. FEMS Microbiol. Lett. **128:**219–228.
28. **Timm, A., and A. Steinbüchel.** 1990. Formation of polyesters consisting of medium-chain-length 3-hydroxyalkanoic acids from gluconate by *Pseudomonas aeruginosa* and other fluorescent pseudomonads. Appl. Environ. Microbiol. **56:**3360–3367.
29. **Wilde, E.** 1962. Untersuchungen über Wachstum und Speicherstoffsynthese von *Hydrogenomonas*. Arch. Mikrobiol. **43:**109–137.
30. **Wilkemeyer, M. F., A. M. Crane, and F. D. Ledley.** 1990. Primary structure and activity of mouse methylmalonyl-CoA mutase. Biochem. J. **271:**449–455.
31. **Williams, D. R., A. J. Anderson, E. A. Dawes, and D. F. Ewing.** 1994. Production of a co-polyester of 3-hydroxybutyric acid and 3-hydroxyvaleric acid from succinic acid by *Rhodococcus ruber*: biosynthetic considerations. Appl. Microbiol. Biotechnol. **40:**717–723.
32. **Yao, W., Y. Yang, and J. Chiao.** 1994. Cloning vector for *Nocardia* spp. Curr. Microbiol. **29:**223–227.