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Megaplasmid and Chromosomal Loci for the PHB Degradation Pathway in *Rhizobium (Sinorhizobium) meliloti*

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ABSTRACT

Chromosomal and megaplasmid loci that affect the poly-3-hydroxybutyrate (PHB) degradation pathway in *Rhizobium meliloti* were identified. A clone that restores the ability of certain *R. meliloti* mutants with defined deletions in megaplasmid pRmeSU47b to use 3-hydroxybutyrate or acetoacetate as the sole carbon source was isolated from a cosmid library of *R. meliloti* genomic DNA. Tn*5* insertion mutagenesis, followed by merodiploid complementation analysis, demonstrated that the locus consists of at least four transcriptional units, *bhbA-D.* We also identified loci involved in 3-hydroxybutyrate and/or acetoacetate utilization by screening for mutants that had lost the ability to use 3-hydroxybutyrate as the sole carbon source while retaining the ability to use acetate (thus ensuring an intact glyoxylate cycle and gluconeogenic pathway). These mutants fell into four classes, as determined by replicon mobilization experiments and genetic linkage in phage transduction; one class corresponded to the *bhb* locus on pRmeSU47b, two classes mapped to different regions on the chromosome and the fourth, *bdhA,* represented by a single mutant, mapped to another pRmeSU47b locus, near *bacA.* The *bdhA* mutant is deficient in 3-hydroxybutrate dehydrogenase activity.

THE polyester poly-3-hydroxybutyrate (PHB) may play a role in the Rhizobium-legume symbiosis. PHB is a bacterial carbon storage compound that accumulates in the cell under conditions where growth is limited but excess carbon supply is available (ANDERSON and DAWES 1990, see Figure 1). It acts as a carbon and electron sink and allows bacterial cells to respond more favorably to starvation and other stress conditions (TAL and OKON 1985). In the absence of an exogenous carbon source, intracellular PHB stores can be used. In PHB synthesis, two molecules of acetyl-CoA are condensed to acetoacetyl-CoA, catalyzed by biosynthetic 3-ketothiolase (encoded by *phbA*) (PEOPLES *et al.* 1987). Acetoacetyl-CoA is then reduced to 3-hydroxybutyryl-CoA by acetoacetyl-CoA reductase (encoded by *phbB*) (PEOPLES and SINSKEY 1989a,b). The 3-hydroxybutyryl-CoA monomer is polymerized by PHB synthase (*phbC*) (SCHUBERT *et al.* 1988; SLATER *et al.* 1988; PEOPLES and SINSKEY 1989c; STEINBÜCHEL and SCHUBERT 1989). The biosynthetic enzymes are constitutively produced, suggesting that regulation occurs at the enzyme level (HAYWOOD *et al.* 1988a,b; 1989). The PHB synthesis genes have recently been cloned from *Rhizobium meliloti* strain 41 (TOMBOLINI *et al.* 1995), and analysis indicates that they are not encoded by a single operon, in contrast to the single operon arrangement in *Alcaligenes eutrophus* (PEOPLES and SINSKEY 1989b,c).

While the synthetic half of the PHB cycle is currently being analyzed genetically in several different organisms, relatively little is known about the genetics of PHB degradation. Biochemical studies indicate that the first step of PHB degradation is depolymerization by PHB depolymerase to form 3- hydroxybutyrate, which is then oxidized to acetoacetate by 3-hydroxybutyrate dehydrogenase. Acetoacetate is converted to acetyl-CoA in two steps involving the enzymes CoA transferase and degradative 3-ketothiolase, and the acetyl-CoA is metabolized via the tricarboxylic acid cycle and glyoxylate shunt (SENIOR and DAWES 1973). Apart from the discovery of the *phbD* gene that encodes intracellular PHB depolymerase within the PHB synthetic gene cluster in some organisms (HUISMAN *et al.* 1991), the genetics of the PHB degradative pathway have not been investigated in any organism to date. *Escherichia coli* does not accumulate PHB, and while *E. coli* cells cannot metabolize 3-hydroxybutyrate, they can metabolize acetoacetate, and the genetics of acetoacetate catabolism has been studied in this organism. The *ato* operon consists of three genes, *atoD, atoA* and *atoB,* expressed as a single transcript under the positive control of the *atoC* gene product (JENKINS and NUNN 1987b). Expression is induced in the presence of acetoacetate (PAULI and OVERATH 1972). *atoD* and *atoA* encode the two subunits of acetoacetyl-CoA transferase, and the distal *atoB* encodes degradative ketothiolase (JENKINS and NUNN 1987a).

PHB may be an important carbon source for soil microorganisms, since extracellular PHB can be used as a carbon source to support growth of many bacteria. For example, BRIESE *et al.* (1994) found that almost 10% of aerobic soil bacteria isolated on nutrient broth

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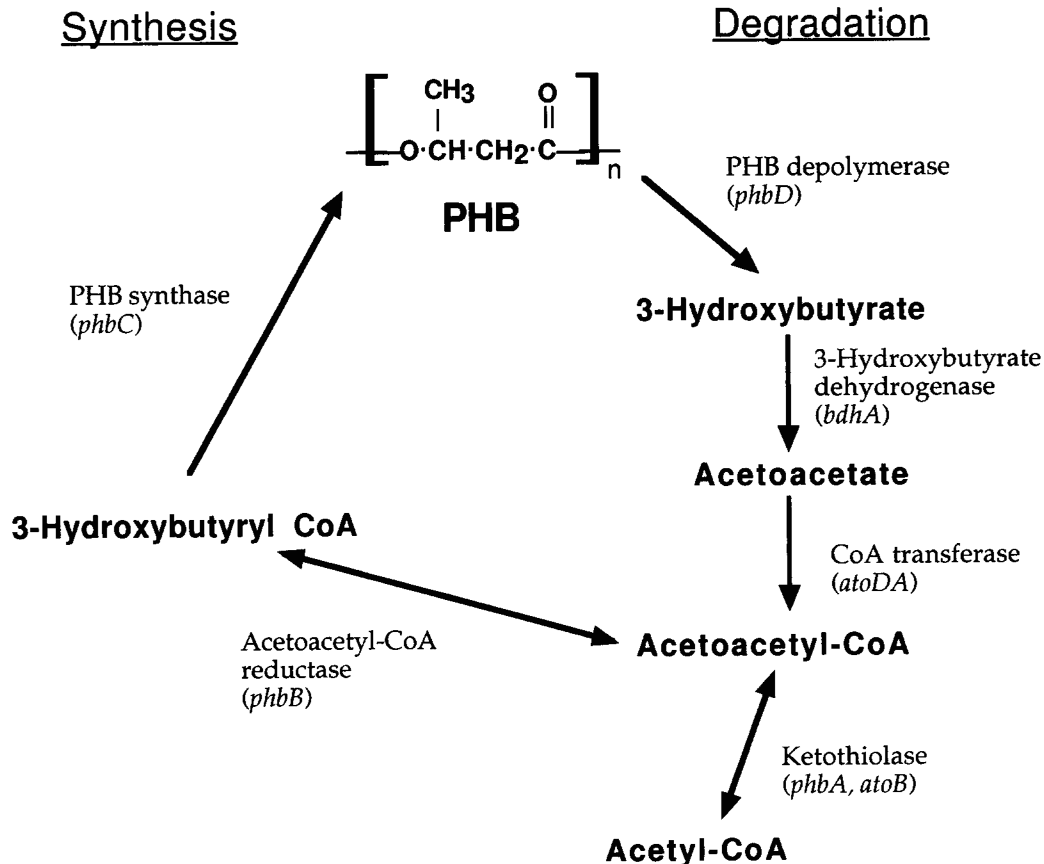

FIGURE 1.—The pathway of PHB synthesis and degradation, with known enzymes and genes indicated.

were able to use PHB. The polymer is first depolymerized by an extracellular PHB depolymerase (a different enzyme than the intracellular *phbD*-encoded enzyme) (NAKAYAMA *et al.* 1985; JENDROSSEK *et al.* 1993; MÜLLER and JENDROSSEK 1993), and the liberated monomer 3-hydroxybutyrate is taken up by the cell. The monomer 3-hydroxybutyrate may also be made available to other organisms that do not themselves possess extracellular depolymerase activity.

In the developing alfalfa root nodule, invasion of nodule tissue by *R. meliloti* cells occurs by bacterial cell division at the growing end of infection threads (GAGE *et al.* 1996). Although PHB and other related polyhydroxyalkanoates can accumulate to high levels in free-living *R. meliloti* cells (>50% of cell dry weight; TOMBOLINI and NUTI 1989) and PHB deposits are observed in bacteria within the infection thread, they are not seen in *R. meliloti* cells that have been released into the plant cell nor in mature bacteroids (PAAU *et al.* 1980; HIRSCH *et al.* 1982; HIRSCH *et al.* 1983). Release of bacteria from the infection threads was correlated with the disappearance of PHB granules. Presence of PHB in cells within the infection thread and its apparent depletion as invasion progresses raises the possibility that PHB that has accumulated before infection commences is used as a carbon and energy source to support proliferation within the infection thread, where supplies from the plant may be limited. Since *dctA* mutants of *R. meliloti*, which are deficient in the uptake of C_4-dicarboxylates, can infect and develop into bacteroids, the C_4-dicarboxylates that support N_2 fixation by the bacteroid are probably not important for growth within the infection thread (WATSON *et al.* 1988; ENGELKE *et al.* 1989), and therefore some other carbon source must be used before the onset of N_2 fixation. The carbon sources that fuel proliferation within the infection thread have not yet been identified (FINAN *et al.* 1991). PHB synthesis mutants of *R. meliloti* have been described, but these were not found to be affected in symbiosis with alfalfa (POVOLO *et al.* 1994).

In contrast with *R. meliloti* bacteroids, which do not accumulate PHB, PHB does accumulate in some other types of bacteroids. For example, PHB accumulation has been observed in *Bradyrhizobium japonicum* bacteroids even under conditions of high nitrogenase activity (MCDERMOTT *et al.* 1989). In one study, a *B. japonicum* mutant that was isolated by nitrous acid treatment was unable to fix N_2 and was observed to form nodules that contained bacteroids that accumulated PHB to only 10% of the wild-type level (SIKORA *et al.* 1994), suggesting a role for PHB in N_2 fixation. Others have proposed a role for bacteroid PHB in the protection of nitrogenase enzyme from oxygen during periods of darkness, by providing reducing power for the maintenance of the O_2 diffusion barrier in the absence of photosynthesis (BERGERSEN *et al.* 1991). Other possible

roles for bacteroid PHB deposits are to aid in the recovery of bacteroids on their release into the soil following nodule senescence (KLUCAS 1975; SUTTON 1983) and to increase the survival of bacteria in the soil and rhizosphere. *R. etli* bacteroids also accumulate PHB in symbiosis, and PHB synthesis mutants of *R. etli* exhibit enhanced symbiotic N_2 fixation (CEVALLOS *et al.* 1996).

Our studies of the genetics of PHB metabolism were initiated with the discovery that deletion of a specific region of megaplasmid pRmeSU47b of *R. meliloti* resulted in the inability to use 3-hydroxybutyrate or acetoacetate as a sole carbon source but did not affect the ability to use acetate (CHARLES and FINAN 1991, see Figure 2). The locus was designated *bhb*, and the phenotype suggested that the metabolic defect was specific to the PHB and fatty acid degradation pathway and probably did not affect the glyoxylate shunt or gluconeogenesis. As the first steps in a long-term effort toward the complete characterization of the PHB degradation pathway in Rhizobium, we wished to determine the nature of the megaplasmid pRmeSU47b locus identified in the megaplasmid deletion studies and also to identify other loci that may be important for PHB degradation. Here we describe the identification of PHB degradation pathway loci by genetic means.

MATERIALS AND METHODS

Bacterial strains, plasmids and transposons: Bacterial strains and plasmids are listed in Table 1. The construction of new strains is described in the text. All *R. meliloti* strains are in the Rm1021 (SU47 *str-21*, streptomycin resistant, Sm^r) background. Bacterial culture in LB and TY complex media and modified M9 minimal salts medium and antibiotic selection were carried out as previously described (CHARLES and FINAN 1991). Modified M9 minimal salts medium was supplemented with glucose, DL 3-hydroxybutyrate (sodium salt), acetoacetate (lithium salt) or acetate (sodium salt) at 15 mM except where otherwise indicated.

Genetic techniques: Transposon Tn*5* (encoding neomycin resistance, Nm^r) mutagenesis of *R. meliloti* strain Rm1021, replacement of Tn*5* insertions with Tn*5*-233 (encoding gentamicin-spectinomycin resistance, Gm^rSp^r), identification of complementing recombinant plasmids from the Rm1021 cosmid library (FRIEDMAN *et al.* 1982), isolation of Tn*5* insertions on IncP plasmids, homogenotization of these insertions (using plasmid pPH1JI), conjugation of IncP plasmids between *R. meliloti* and *E. coli* (using the mobilizing strain MT616) and transduction between *R. meliloti* strains using phage ΦM12 (FINAN *et al.* 1984) were carried out as previously described (CHARLES and FINAN 1990; CHARLES *et al.* 1991). Each newly isolated insertion mutant was checked for cotransduction of phenotype and the antibiotic resistance of the transposon. To map the locations of chromosomal Tn*5*-233 insertions, we used the Tn*5-mob* chromosomal insertions described by KLEIN *et al.* (1992), using donor strains in the RCR2011 strain background constructed by T. FINAN and ourselves (T. CHARLES, unpublished data; T. FINAN, unpublished data). Each Tn*5*-233 insertion was transduced into each of six Tn*5-mob* mapping strains. After streak purification, these transductants were conjugated with Rm5000 (rifampicin resistant, Rf^r), using MT616 as the mobilizing strain. Late log phase cultures were mixed, incubated on TY agar overnight, suspended in saline and spread on selective media. Transconjugants arising after selection for $Rf^rGm^rSp^r$ were scored and divided by the number of Rf^r recipient cells to determine conjugation frequency. For megaplasmid conjugation mapping experiments, the Tn*5* insertions were transduced into the pRmeSU47a and pRmeSU47b Tn*5*-11 (Gm^rSp^r, *oriT*) strains Rm5320 and RmF123, respectively, by selecting for Nm^r. The resulting double insertion strains were conjugated with *Agrobacterium tumefaciens* GMI9023, and cotransfer of Nm^r and Gm^rSp^r was indicative of a megaplasmid location of the Tn*5* insertion. Restriction digests and agarose gel electrophoresis were carried out using standard methods (AUSUBEL *et al.* 1991).

Growth kinetics: Late log phase TY cultures were washed once in 0.85% saline, and 0.15 ml was subcultured into 5 ml M9 supplemented with the appropriate carbon source in a 16 × 150 mm culture tube and placed vertically in a rack in a shaking incubator set at 200 rpm, 30°. Growth was followed by measuring absorbance at 600 nm. Upon completion of the growth test, culture purity was checked by streaking on TY agar.

Biochemical assay and enzyme activity staining: Cell cultures were grown in TY broth overnight (*R. meliloti*) or in M9 containing 15 mM 3-hydroxybutyrate for 4 days (*A. tumefaciens*) at 30° in a shaker at 200 rpm. The purity of the cultures was checked by streaking from them onto TY agar plates. Cells were collected by centrifugation, the pellets were washed twice in a buffer consisting of 20 mM Tris-Cl pH 7.8, 1 mM $MgCl_2$ and were stored at −20°. Before sonication, pellets were suspended in 4 ml of sonication buffer (20 mM Tris-Cl pH 7.8, 1 mM $MgCl_2$, 10% glycerol and 10 mM β-mercaptoethanol) per gram cell wet weight and thereafter kept on ice. The cells were disrupted by sonication (Ultrasonics Sonifier Cell Disruptor Model W185-D). Cell debris was removed by centrifugation (SS34 rotor, 12,000 rpm, 20 min). The resultant cell-free extracts were stored at −70°. Protein concentration was determined by the method of BRADFORD (1976), using bovine serum albumin as standard.

The activity of 3-hydroxybutyrate dehydrogenase (E.C. 1.1.1.30) was assayed according to the method of BERGMEYER *et al.* (1967). Cell extract (50–100 μl) was added to a reaction mixture consisting of 33 mM Tris-Cl pH 8.4, 1.8 mM NAD^+, 22 mM DL 3-hydroxybutyrate (sodium salt) in a total volume of 1.55 ml. The conversion of NAD^+ to NADH was determined by monitoring the change in absorbance at 340 nm using a Beckman DU70 spectrophotometer. Negative controls were omission of 3-hydroxybutyrate substrate, NAD^+ or extract, and no activity was detected under these conditions. Specific activity is expressed as nmol/min/mg protein. Values presented are the means of three assays.

Nondenaturing PAGE was also used to detect 3-hydroxybutyrate dehydrogenase activity. Cell extracts equivalent to 75 μg total protein were electrophoresed at 4° through a native polyacrylamide gel (4% stacking, 7.5% resolving) on a Protean mini gel apparatus (BioRad) using Tris Glycine (pH 8.3) buffer at 25 mA for 1 hr. Activity of 3-hydroxybutyrate dehydrogenase was detected as described by SELANDER *et al.* (1986) after incubation in a staining solution comprised of 0.2 M Tris-Cl pH 8.0, 2 mg/ml DL 3-hydroxybutyrate, 4 mM $MgCl_2$, 4 mg/ml NaCl, 0.2 mg/ml NAD, 0.25 mg/ml (4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and 0.1 mg/ml phenazine methosulphate (PMS). For detection of unidentified dehydrogenase activity bands, 3-hydroxybutyrate was omitted from the staining solution.

RESULTS

Cloning and analysis of megaplasmid *bhb* locus: The pRmeSU47b 190-kilobase (kb) deletion strain RmF726

TABLE 1

Bacterial strains, plasmids and transposons

Strain or plasmid	Relevant characteristics	Source, reference or construction
Rhizobium meliloti		
RCR2011	=SU47 wild type	Rothamsted Experimental Station
Rm1021	SU47 *str-21*	MEADE *et al.* (1982)
Rm5000	SU47 *rif-5*	FINAN *et al.* (1984)
RCR2011 derivatives		
RmH347	Ω601::Tn*5-mob*	T. FINAN, unpublished results; KLEIN *et al.* (1992)
RmH348	Ω602::Tn*5-mob*	T. FINAN, unpublished results; KLEIN *et al.* (1992)
RmH349	Ω611::Tn*5-mob*	T. FINAN, unpublished results; KLEIN *et al.* (1992)
RmH350	Ω612::Tn*5-mob*	T. FINAN, unpublished results; KLEIN *et al.* (1992)
RmH353	Ω637::Tn*5-mob*	T. FINAN, unpublished results; KLEIN *et al.* (1992)
Rm11151	Ω615::Tn*5-mob*	T. CHARLES, unpublished results; KLEIN *et al.* (1992)
Rm1021 derivatives		
Rm5282	Ω5015::Tn*5*-132	FINAN (1988)
Rm5320	pRmeSU47aΩ30::Tn*5*-11	FINAN *et al.* (1986)
Rm5404	Ω5040::Tn*5*-233	CHARLES and FINAN (1990)
Rm8386	*phoA bacA386*::Tn*phoA*	LONG *et al.* (1988)
RmF123	Ω5047::Tn*5*-11	CHARLES (1991)
RmF581	Ω5149::Tn*5*-233 Ω5072::Tn*5*-132	CHARLES (1991)
RmF726	ΔΩ5149-5079::Tn5-233	CHARLES and FINAN (1991)
Rm11035	*bhbA1*::Tn*5* (pPH1JI)	Homogenote of pTC322Ω1::Tn*5*
Rm11062	*bhbA1*::Tn*5*	Homogenote of pTC322Ω1::Tn*5*
Rm11064	*bhbB6*::Tn*5*	Homogenote of pTC322Ω6::Tn*5*
Rm11066	*bhbC4*::Tn*5*	Homogenote of pTC322Ω4::Tn*5*
Rm11067	*bhbB11*::Tn*5*	Homogenote of pTC322Ω11:Tn*5*
Rm11068	*bhbD12*::Tn*5*	Homogenote of pTC322Ω12::Tn*5*
Rm11070	*bhbA14*::Tn*5*	Homogenote of pTC322Ω14::Tn*5*
Rm11073	*bhbC20*::Tn*5*	Homogenote of pTC322Ω20:Tn*5*
Rm11085	*bhbA1*::Tn*5*-233	Tn*5*-233 replacement of Rm11062
Rm11089	*bhbC24*::Tn*5*	Homogenote of pTC322Ω24::Tn*5*
Rm11104	*age-1*::Tn*5*	This study
Rm11105	*aau-1*::Tn*5*	This study
Rm11107	*bdhA1*::Tn*5*	This study
Rm11110	*aau-3*::Tn*5*	This study
Rm11116	*bhbA16*::Tn*5*	This study
Rm11133	*aau-6*::Tn*5*	This study
Rm11134	*aau-7*::Tn*5*	This study
Rm11135	*aau-8*::Tn*5*	This study
Rm11136	*bhbA36*::Tn*5*	This study
Rm11139	*bhbC39*::Tn*5*	This study
Rm11143	*age-1*::Tn*5*-233	This study
Rm11144	*aau-1*::Tn5-233	This study
Rm11145	*bdhA1*::Tn5-233	This study
Rm11160	*aau-7*::Tn5-233	This study
Rm11161	*aau-8*::Tn5-233	This study
Rm11164	*aau-3*::Tn5-233	This study
Rm11167	*aau-6*::Tn*5*-233	This study
Agrobacterium tumefaciens		
GMI9023	C58 cured of pTiC58 and pAtC58, Sm^{r}Nm^r	ROSENBERG and HUGUET (1984)
At125	GMI9023, pRmeSU47bΩ5007::Tn*5-oriT*	FINAN *et al.* (1986)
Escherichia coli		
MT607	*pro-82 thi-1 hsdR17* *supE44 recA56*	FINAN *et al.* (1986)
MT614	MT607ΩTn*5*	T. FINAN, unpublished results
MT616	MT607(pRK600); mobilizing strain	FINAN *et al.* (1986)
Plasmids		
pTC322	pLAFR1 clone, Tc^r, complements *bhb* mutants	This study
pPH1JI	IncP Gm^{r}Sp^{r}Cm^r	BERINGER *et al.* (1978)

Abbreviations are as follows: Gm, gentamicin; Nm, neomycin; Ot, Oxytetracycline; Rf, Rifampicin; Sm, streptomycin; Sp, spectinomycin; Tc, tetracycline. The transposon antibiotic resistance markers are Nm^r (Tn*5*, Tn*5-oriT*, Tn*5-mob*, Tn*phoA*); Gm^{r}Sp^r (Tn*5*-233, Tn*5*-11); Ot^r (Tn*5*-132).

is unable to use either 3-hydroxybutyrate or acetoacetate as the sole carbon source. The locus responsible for this phenotype was previously designated *bhb* (CHARLES and FINAN 1991). The Rm1021 pLAFR1 (tetracycline resistant, Tc^r) cosmid library (maintained in *E. coli* HB101) was introduced into RmF726 by conjugation, and selection was carried out on 3-hydroxybutyrate thiamine minimal medium (the deletion in RmF726 also removes *thi-502*). After streak purification of five colonies on the same medium, the cosmid clones were recovered from the transconjugants by conjugation into *E. coli* MT607, selecting for Tc^r. All five clones had the identical *Eco*RI restriction pattern. One of these clones was designated pTC322, and its ability to complement the deletion strain was confirmed by reintroduction into RmF726 followed by testing for ability of the transconjugants to grow on 3-hydroxybutyrate thiamine minimal medium.

Transposon Tn*5* mutagenesis of pTC322 was carried out in order to localize the *bhb* complementing gene(s) on the cosmid clone. Since pTC322 is mobilizable in *trans* using the mobilizing strain MT616, it was possible to select for transposition of a chromosomal Tn*5* insertion onto pTC322 by mobilizing pTC322 out of *E. coli* strain MT614 (which carries a chromosomal Tn*5* insertion) into the deletion strain RmF726. Transposition of Tn*5* from the chromosome to pTC322 will occur prior to the conjugation event, and the resulting pTC322ΩTn*5* will be available for conjugal transfer to RmF726. Hence, triparental conjugations were carried out, with MT614(pTC322) as donor, MT616 as mobilizer and RmF726 as recipient. Transconjugant colonies arising on TY Sm Nm Tc medium after 4 days incubation were screened directly for inability to grow on 3-hydroxybutyrate thiamine minimal medium, which indicated disruption of the *bhb* gene(s) by Tn*5*. The plasmids in these colonies were then transferred by conjugation into *E. coli* MT607. Subsequently, several of these plasmids were conjugated into Rm1021 and the insertions were recombined into the genome by homogenotization, thus generating insertion mutants. The homogenotes were tested for growth on 3-hydroxybutyrate and acetoacetate, and the clones were characterized by *Eco*RI restriction digest analysis, to determine the fragments carrying the individual insertions (Table 2). The homogenotized Tn*5* insertions were then transduced into Rm1021.

Complementation analysis was done to determine the number of transcriptional units involved in the 3-hydroxybutyrate utilization phenotype of the RmF726 deletion mutant. A series of merodiploid strains was constructed by conjugal transfer of pTC322ΩTn*5* plasmids into individual homogenotes. The merodiploids were tested for growth on 3-hydroxybutyrate (Table 2). The results place the insertions into four clear complementation groups, and this is consistent with the physical data from the restriction digest analysis. Since Tn*5* generates polar mutations, our analysis indicates the existence of at least four transcriptional units on pTC322 that are required for utilization of 3-hydroxybutyrate and acetoacetate as sole carbon source. We designate these loci *bhbA*, *bhbB*, *bhbC* and *bhbD*, represented by the insertion mutations Ω1, Ω6, Ω4 and Ω12, respectively.

Three factor transductional crosses were performed to determine the precise location of these loci within the region of the large megaplasmid deletion. Nm^r was transduced from strain Rm11035 (=*bhbA1*::Tn*5*) into strain RmF581, which carries Tn*5*-233 and Tn*5*-132 (oxytetracycline resistance, Ot^r) insertions at Ω5149 and Ω5072, respectively (CHARLES and FINAN 1990). The linkage data (not shown) indicate that the *bhb* insertion maps near Ω5072 (89% cotransduction, clockwise side) at coordinate 461 kb on the pRmeSU47b transductional linkage map (Figure 2).

Isolation of other 3-hydroxybutyrate utilization mutants by Tn*5* mutagenesis: Tn*5* mutagenesis of the *R. meliloti* genome was carried out to identify other loci necessary for PHB degradation. A total of 4400 Tn*5* mutants was screened for inability to grow on M9 3-hydroxybutyrate agar. Of the 14 putative mutants, five were unable to grow on acetate, acetoacetate or 3-hydroxybutyrate, and these were not studied further. Only one mutant, strain Rm11107, was able to use acetoacetate as the sole carbon source. This is the expected phenotype of a 3-hydroxybutyrate dehydrogenase mutant, and the insertion thus was designated *bdh-1* (beta-hydroxybutyrate dehydrogenase). Eight of the nine remaining mutants were phenotypically similar to the *bhb* mutants, being unable to grow on acetoacetate or 3-hydroxybutyrate, and this phenotype was designated Aau$^-$, for acetoacetate utilization. Three of the eight Aau$^-$ insertions, in strains Rm11116, Rm11136 and Rm11139, were in fact tightly linked in transduction (>95%) to the megaplasmid *bhbA1*::Tn*5*-233 insertion in strain Rm11085. Merodiploid complementation analysis with the pTC322ΩTn*5* plasmids (Table 2) indicated that the insertions in Rm11116 and Rm11136 were in *bhbA*, and the insertion in Rm11139 was in *bhbC*. This left six mutants (five Aau$^-$ plus one Bdh$^-$) in the new 3-hydroxybutyrate utilization loci. Also isolated in this screen was one mutant (Rm11104) that exhibited enhanced growth rate on 3-hydroxybutyrate and acetoacetate. This phenotype was designated Age$^+$ (acetoacetate growth enhanced).

The Tn*5* insertions in each of the unmapped mutants were replaced with Tn*5*-233 and placed into classes by cotransductional linkage. One class consisted of *aau-3*::Tn*5*, *aau-6*::Tn*5*, *aau-7*::Tn*5*, *aau-8*::Tn*5* and the insertion *age-1*::Tn*5*, which caused an enhanced growth rate on 3-hydroxybutyrate and acetoacetate. Transductional linkage of 99–100% was demonstrated between *aau-6*::Tn*5* and Tn*5*-233 insertions of the other members of this cluster, with the exception of *aau-3*::Tn*5*-233, which was 78% linked. The *aau-1*::Tn*5* and *bdh-*

TABLE 2

Restriction digest and complementation analysis of the *bhb* locus of *R. meliloti*

Strain	Chromosomal insertion	*Eco*RI fragment	Class	pTC322::Tn5 insertion							
				1	4	6	11	12	14	20	24
Rm11062	1	3.8	A	−	+	+	+	+	−	+	+
Rm11066	4	7.5	C	+	−	+	+	+	+	−	−
Rm11064	6	7.5	B	+	+	−	−	+	+	+	+
Rm11067	11	7.5	B	+	+	−	−	+	+	+	+
Rm11068	12	4.7	D	+	+	+	+	−	+	+	+
Rm11070	14	3.8	A	−	+	+	+	+	−	+	+
Rm11073	20	7.5	C	+	−	+	+	+	+	−	−
Rm11089	24	0.5	C	+	−	+	+	+	+	−	−
Rm11116	NA	ND	A	−	+	+	+	+	−	+	+
Rm11136	NA	ND	A	−	+	+	+	+	−	+	+
Rm11139	NA	ND	C	+	−	+	+	+	+	−	−

Colony formation was scored after incubation for 10 days on M9 3-hydroxybutyrate. +, colony formed, −, no colony formed; NA, not applicable; ND, not determined.

1::Tn*5* insertions were single members of two other classes.

Growth kinetics of 3-hydroxybutyrate and/or acetoacetate utilization mutants: Carbon source utilization growth tests were carried out for representative mutants in liquid culture to confirm the growth phenotypes observed on agar-solidified media. Growth of all mutants on glucose and acetate was similar to that of the wild-type control (Figure 3A and D), except the *aau-1* strain, which was slightly reduced on acetate. Growth of the *age-1* strain was much greater on 3-hydroxybutyrate than the wild-type control (Figure 3B) and significantly greater on acetoacetate (Figure 3C, doubling times of 8.3 and 15 hr, respectively). The *aau-7* and *bdh-1* strains showed no appreciable growth on 3-hydroxybutyrate, while the growth of the *aau-1* strain was considerably reduced on 3-hydroxybutyrate (Figure 3B). On acetoacetate, growth of the *bdh-1* strain was similar to that of the wild-type control strain (15 hr doubling time), no growth was observed for the *aau-7* strain and growth of the *aau-1* strain was reduced considerably (34 hr doubling time) (Figure 3C).

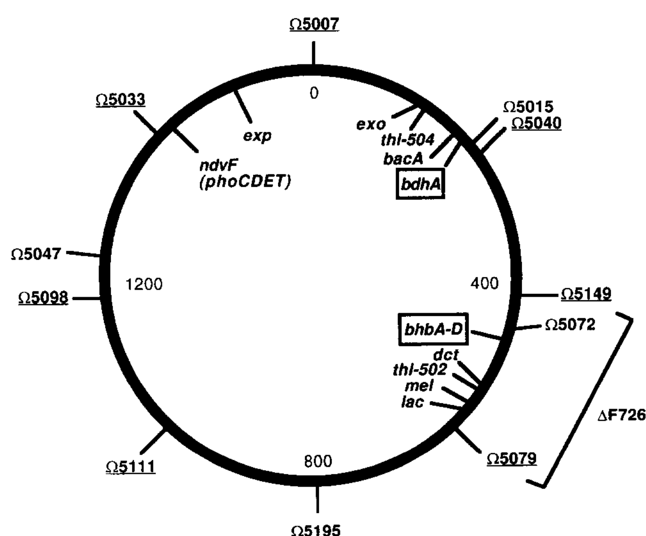

FIGURE 2.—Genetic map of megaplasmid pRmeSU47b based on transductional linkage studies (CHARLES and FINAN 1990; CHARLES *et al.* 1990; CHARLES and FINAN 1991; this study). Numbers in the interior of the circle indicate approximate distance clockwise from Ω5007 in kilobases. The insertions in the eight pRmeSU47b mapping strains (CHARLES 1991) are underlined. The *ndvF* locus has recently been found to comprise four genes, *phoCDET*, which encode an ABC-type transporter for uptake of P_i (BARDIN *et al.* 1996).

Genomic mapping of 3-hydroxybutyrate utilization mutations: Tn*5*-233 insertions representative of each class were mapped using the Tn*5*-*mob* chromosomal mapping strains. Transconjugants were obtained in at least one member of the set for each insertion, except *bdh-1*::Tn*5* (Table 3). The relative chromosomal position for each of the insertions was inferred from the conjugation frequencies, and the insertions were situated in two different regions of the chromosome (Figure 4). Since insertion *bdh-1*::Tn*5*, which causes the phenotype expected of a 3-hydroxybutyrate dehydrogenase mutant, could not be mapped to the chromosome, it was mapped using the megaplasmid Tn*5*-11 mobilization strains (Rm5320, for pRmeSU47a and RmF123, for pRmeSU47b) and *A. tumefaciens* GMI9023 as recipient. Transconjugants only arose when the RmF123 construct was the donor, indicating that *bdh-1*::Tn*5* was on pRmeSU47b. To determine the region of the megaplasmid where *bdh-1*::Tn*5* resided, Nm^r from strain Rm11107 was transduced into each of the eight pRmeSU47b mapping strains, which have Gm^rSp^r insertions (Figure 2, underlined insertions) distributed at ~200-kb intervals on pRmeSU47b (CHARLES 1991). Cotransduction only occured in one case, to Rm5404 (containing Ω5040::Tn*5*-233), indicating that *bdh-1*::Tn*5* and Ω5040 are 35% linked in transduction. Insertion *bdh-1*::Tn*5* was more precisely mapped by three-factor cross to other megaplasmid insertions (data not shown) and was found to be located at coordinate

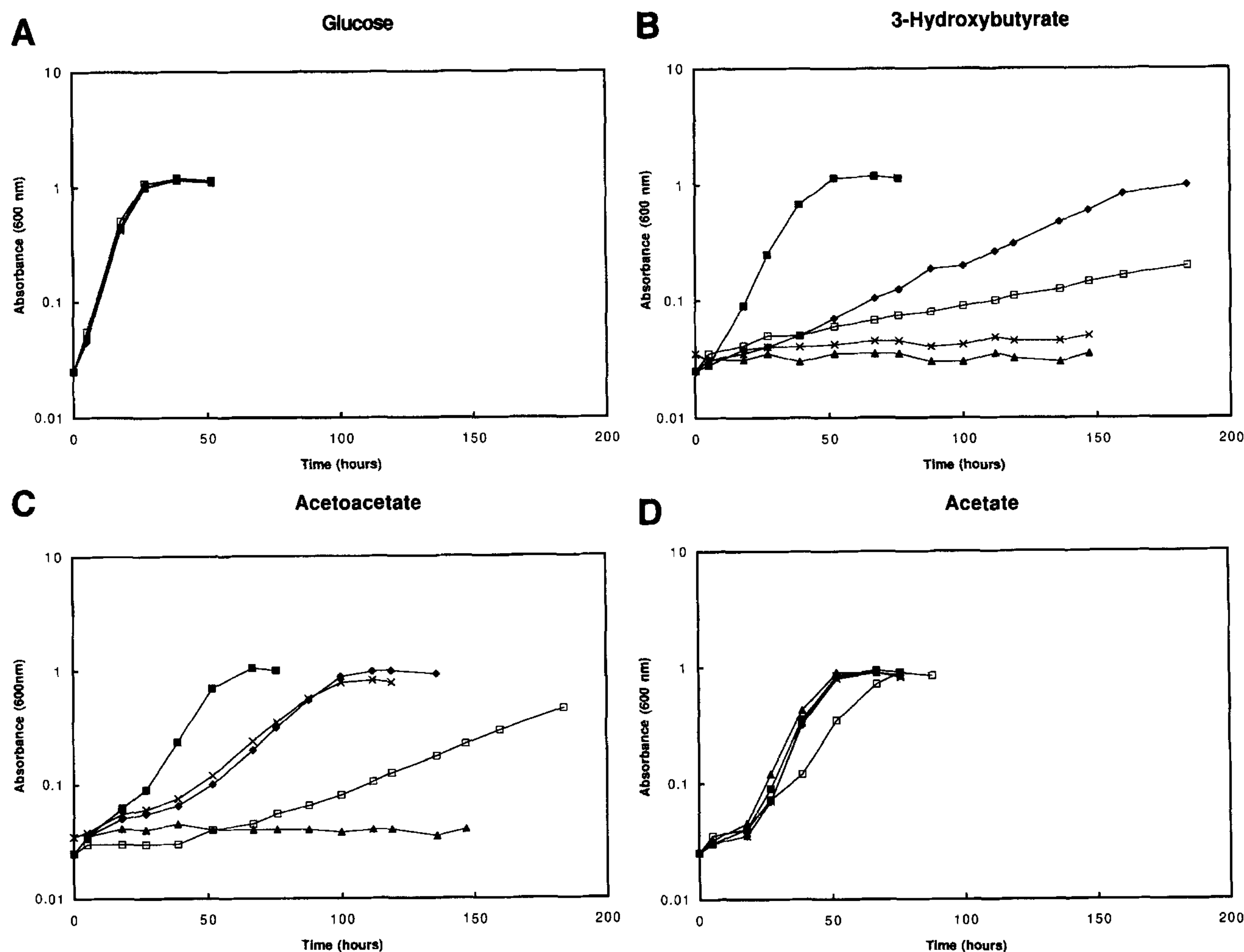

FIGURE 3.—Comparison of the growth kinetics of the wild-type strain and representative mutants on different carbon sources. Strains used were Rm1021 (wild type), Rm11104 (*age-1*::Tn*5*), Rm11105 (*aau-1*::Tn*5*), Rm11107 (*bdh-1*::Tn*5*) and Rm11134 (*aau-7*::Tn*5*). (A) 10 mM glucose; (B) 15 mM 3-hydroxybutyrate; (C) 15 mM acetoacetate; (D) 30 mM acetate. The growth of each culture was followed for 184 hr or until stationary phase was reached. ◆, wild type; ■, *age-1*; ▲, *aau-7*; ×, *bdh-1*; □, *aau-1*.

200 kb on the pRmeSU47b transductional linkage map (Figure 2). It is in fact fairly tightly linked (92% cotransduction, clockwise side) to *bacA*, a gene that is required for bacteroid development (GLAZEBROOK *et al.* 1993). A *bacA* mutant Rm8386 was able to grow on 3-hydroxybutyrate and exhibited 3-hydroxybutyrate dehydrogenase activity (data not shown).

3-hydroxybutyrate dehydrogenase activity is megaplasmid pRmeSU47b-encoded: The phenotype associated with insertion *bdh-1*::Tn*5*, that is, the inability to utilize 3-hydroxybutyrate as the sole carbon source, while retaining the ability to grow on acetoacetate, would be expected for a 3-hydroxybutyrate dehydrogenase mutant. To test this, a cell-free extract of strain Rm11107 was prepared and was assayed for 3-hydroxybutyrate dehydrogenase enzyme activity. No activity was detected, compared to 44 nmol/min/mg protein for the Rm1021 parental control. Examination of enzyme

TABLE 3

Conjugal mapping of 3-hydroxybutyrate utilization mutations in *R. meliloti*

	No. of recombinants/10^8 recipient cells from the following Tn*5*-*mob* inserts					
Mutant	Ω601	Ω602	Ω611	Ω612	Ω615	Ω637
aau-1	842	0	0	0	0	3330
aau-6	0	0	1178	0	378	0
bdh-1	0	0	0	0	0	0

Rm5000 (Rf^r) was the recipient strain. *E. coli* strain MT616 provided mobilization functions. Recombinants were selected on TY agar containing rifampicin, gentamicin and spectinomycin.

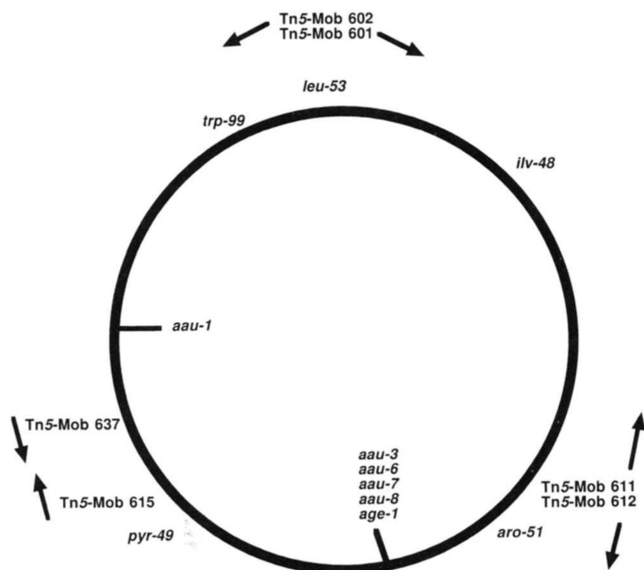

FIGURE 4.—Genetic map of the *R. meliloti* chromosome, showing locations of acetoacetate utilization loci. Arrows indicate positions and orientations of Tn*5-mob* insertions. The positions of the auxotrophic markers are from KLEIN *et al.* (1992). The positions of the mapped *aau* loci are approximate, based on conjugation and transduction linkage data (Table 3).

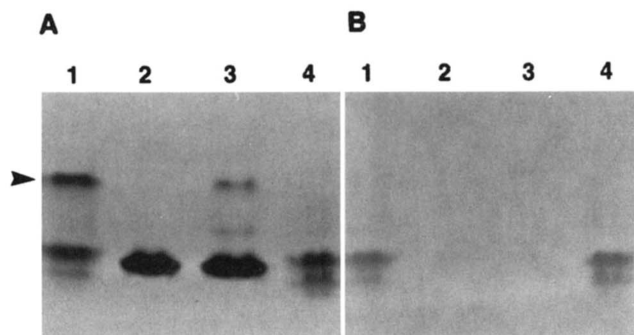

FIGURE 5.—Analysis of 3-hydroxybutyrate dehydrogenase activity by native gel electrophoresis. (A) With 3-hydroxybutyrate substrate; (B) without 3-hydroxybutyrate substrate. Lane 1, *R. meliloti* Rm1021 (wild type); lane 2, *A. tumefaciens* GMI9023; lane 3, *A. tumefaciens* At125; lane 4, *R. meliloti* Rm11107 (*bdh-1* Tn*5*).

activity on native gels confirmed that 3-hydroxybutyrate dehydrogenase activity could not be detected for strain Rm11107 (Figure 5A, lane 4), although the wild type and the mutant both exhibited similar levels of the unidentified dehydrogenase activity (Figure 5B, lanes 1 and 4) (CHARLES *et al.* 1990). These results demonstrate that Rm11107 is a 3-hydroxybutyrate dehydrogenase mutant. It was possible that insertion *bdh-1*::Tn*5* affected 3-hydroxybutyrate dehydrogenase activity without actually disrupting the structural gene. We therefore examined cell-free extracts of *A. tumefaciens* strain At125, which contains pRmeSU47b, for 3-hydroxybutyrate dehydrogenase activity on native gels to confirm that the structural gene was carried by the megaplasmid. The At125 extract had an additional band of 3-hydroxybutyrate dehydrogenase activity, of the same mobility as the *R. meliloti* band, which was not present in the parental *A. tumefaciens* strain GMI9023 (Figure 5A, lanes 2 and 3). A second additional band of mobility intermediate to the *R. meliloti* and *A. tumefaciens* 3-hydroxybutyrate dehydrogenase bands may represent hybrid enzymes resulting from mixing of the *R. meliloti* and *A. tumefaciens* subunits. We have designated the 3-hydroxybutyrate dehydrogenase locus *bdhA* in accord with the designation of the eukaryal 3-hydroxybutyrate dehydrogenase structural genes (CHURCHILL *et al.* 1992; MARKS *et al.* 1992).

DISCUSSION

We have identified two chromosomal and two megaplasmid loci that are required for utilization of 3-hydroxybutyrate and/or acetoacetate as the sole carbon and energy source. As the immediate product of PHB depolymerization is 3-hydroxybutyrate, and assuming that 3-hydroxybutyrate of extracellular origin is metabolized in a manner similar to the intracellular product of PHB depolymerization, these loci must be affected in the PHB degradation pathway. The failure of most of the 3-hydroxybutyrate utilization mutants to grow on acetoacetate is genetic confirmation that 3-hydroxybutyrate is metabolized through acetoacetate in *R. meliloti.* The only mutant that was unable to grow on 3-hydroxybutyrate, while retaining the ability to grow on acetoacetate, was found to lack 3-hydroxybutyrate dehydrogenase activity. Detection of apparently hybrid *R. meliloti/A. tumefaciens* 3-hydroxybutyrate dehydrogenase suggests a multisubunit nature for the *R. meliloti* and *A. tumefaciens* enzymes, perhaps similar to the tetramer nature of the *Azospirillum brasilense* enzyme (TAL *et al.* 1990).

Two distinct regions of the megaplasmid pRmeSU47b contain 3-hydroxybutyrate degradation loci. It should be noted that the 3-hydroxybutyrate dehydrogenase locus lies in a region of the megaplasmid that has been recalcitrant to deletion, which explains why it was not identified via the pRmeSU47b deletion studies (CHARLES and FINAN 1991). Determination of whether the 3-hydroxybutyrate dehydrogenase locus contains the structural gene for the enzyme awaits DNA sequence analysis. The proximity of *bdhA* to *bacA* encourages speculation regarding a role for 3-hydroxybutyrate dehydrogenase in symbiosis or for *bacA* in 3-hydroxybutyrate metabolism. It has been observed that *bacA* mutant cells are not depleted of PHB deposits upon release from the infection thread, whereas wild-type cells are, suggesting that the metabolism of PHB during infection is influenced by the *bacA* mutation (GLAZEBROOK *et al.* 1993). Considering that *bacA* is not required for growth on 3-hydroxybutyrate, any relationship between 3-hydroxybutyrate dehydrogenase and *bacA* may be indirect.

To our knowledge, the only previous report of the

generation of a bacterial 3-hydroxybutyrate dehydrogenase mutant is that of EMERICH (1984). In that case, a *B. japonicum* mutant that lacked 3-hydroxybutyrate dehydrogenase activity was isolated by UV mutagenesis. Interestingly, this mutant was also affected in symbiotic efficiency on soybean, being unable to fix N_2. It is important to emphasize that *B. japonicum* bacteroids in soybean nodules differ from *R. meliloti* bacteroids in alfalfa nodules with respect to PHB content. While the *B. japonicum* bacteroids accumulate PHB during N_2 fixation, the *R. meliloti* bacteroids do not accumulate PHB. Although *R. meliloti* PHB synthesis mutants have been reported to be fully effective in symbiosis with alfalfa (POVOLO *et al.* 1994), the symbiotic phenotype of our degradation mutants remains to be determined. We will now be able to use the *bdhA* and other PHB degradation pathway mutants to study the regulation of PHB accumulation in the bacteroid and perhaps determine why *R. meliloti* bacteroids do not accumulate PHB, while other types of bacteroids do accumulate PHB.

The nature of the mutation that results in increased growth rate on 3-hydroxybutyrate and acetoacetate (Age^+ phenotype) is not apparent. The simplest explanation is that the mutation affects the production of a negative regulatory element, causing derepression of the production of an enzyme that is limiting for conversion of acetoacetate to acetyl-CoA (*i.e.*, CoA transferase or ketothiolase). It is tempting to speculate that this gene is directly involved in the regulation of the adjacent (99% cotransductional linkage) *aau* locus, but this conclusion would be premature if based solely on the available evidence.

Previous biochemical studies indicated that degradation of the PHB depolymerization product 3-hydroxybutyrate to acetyl-CoA involved three enzymes: 3-hydroxybutyrate dehydrogenase, CoA transferase and degradative ketothiolase (SENIOR and DAWES 1973). In *E. coli*, the only organism in which genetic studies of acetoacetate utilization have been done, the latter two enzymes are encoded in a single operon (PAULI and OVERATH 1972). Thus, it was expected that only a few genes would be involved in acetoacetate utilization in *R. meliloti*. However, we have found one megaplasmid locus, comprised of at least four transcriptional units, and two chromosomal loci, for acetoacetate utilization. The products of the expression of these loci are not known, but DNA sequence analysis should give an indication of the functional nature of these genes. The megaplasmid location of several of the genes in this pathway underscores the importance of pRmeSU47b in overall carbon metabolism in *R. meliloti*. It is now clear that the factors that influence the operation of the PHB degradation pathway are more complex than we had originally envisioned.

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