JOURNAL OF BACTERIOLOGY, Mar. 2002, p. 1571–1577 Vol. 184, No. 6
0021-9193/02/$04.00+0 DOI: 10.1128/JB.184.6.1571–1577.2002


# Identification of an Acetoacetyl Coenzyme A Synthetase-Dependent Pathway for Utilization of L-(+)-3-Hydroxybutyrate in *Sinorhizobium meliloti*

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Received 11 September 2001/Accepted 4 December 2001

**D-(−)-3-Hydroxybutyrate (DHB), the immediate depolymerization product of the intracellular carbon store poly-3-hydroxybutyrate (PHB), is oxidized by the enzyme 3-hydroxybutyrate dehydrogenase to acetoacetate (AA) in the PHB degradation pathway. Externally supplied DHB can serve as a sole source of carbon and energy to support the growth of *Sinorhizobium meliloti*. In contrast, wild-type *S. meliloti* is not able to utilize the L-(+) isomer of 3-hydroxybutyrate (LHB) as a sole source of carbon and energy. In this study, we show that overexpression of the *S. meliloti acsA2* gene, encoding acetoacetyl coenzyme A (acetoacetyl-CoA) synthetase, confers LHB utilization ability, and this is accompanied by novel LHB-CoA synthetase activity. Kinetics studies with the purified AcsA2 protein confirmed its ability to utilize both AA and LHB as substrates and showed that the affinity of the enzyme for LHB was clearly lower than that for AA. These results thus provide direct evidence for the LHB-CoA synthetase activity of the AcsA2 protein and demonstrate that the LHB utilization pathway in *S. meliloti* is AcsA2 dependent.**

Poly-3-hydroxybutyrate (PHB), a bacterial intracellular reserve of carbon and reducing energy, accumulates when a nutrient other than carbon is limiting for growth, as reviewed by Madison and Huisman (18). Based on biochemical evidence, the metabolism of PHB has been proposed to be a cyclical process, comprising the pathways for synthesis and degradation of PHB. Synthesis of PHB occurs when excess carbon, in the form of acetyl coenzyme A (acetyl-CoA), is condensed via a ketothiolase (EC 2.3.1.9; encoded by the *phbA* gene) enzyme to generate acetoacetyl-CoA. Reduction of acetoacetyl-CoA to D-3-hydroxybutyryl-CoA is carried out by an NADP-dependent acetoacetyl-CoA reductase (EC 1.1.1.36; encoded by the *phbB* gene) (21). The PHB synthase enzyme (encoded by the *phbC* gene) catalyzes the final polymerization of D-3-hydroxybutyryl-CoA (21, 22).

Degradation of PHB is initiated by the depolymerization of the PHB granules, catalyzed by PHB depolymerase. A central enzyme in the PHB cycle is D-(−)-3-hydroxybutyrate dehydrogenase (BDH; EC 1.1.1.30; encoded by *bdhA*), which catalyzes the oxidation of the immediate PHB depolymerization product D-(−)-3-hydroxybutyrate (DHB) to acetoacetate (AA). The AA product of this reaction is activated to acetoacetyl-CoA by the enzyme acetoacetyl-CoA synthetase (AACS; EC 6.2.1.16), encoded by *acsA2* in *Sinorhizobium meliloti* (5, 7, 15). The final step in the degradation portion of the PHB cycle yields two molecules of acetyl-CoA from one molecule of acetoacetyl-CoA via ketothiolase (EC 2.3.1.9). *S. meliloti acsA2* mutants are able to grow with acetate as a carbon source but not with AA; *acsA1* (7, 15) mutants, which do not produce acetyl-CoA synthetase (EC 6.2.1.1), are unable to grow on acetate but are able to grow on AA (20). While wild-type *S. meliloti* is able to grow using either DHB or AA, it is unable to use L-(+)-3-hydroxybutyrate (LHB) as a sole source of carbon.

Although there is a considerable body of research on the biochemistry and genetics of bacterial PHB synthesis, apart from our recent studies on the PHB degradation pathway of *S. meliloti* (1, 5, 8), not much is known about the genetics of the bacterial PHB degradation pathway(s) or assimilation of 3-hydroxybutyrate (3-HB). Our studies of the PHB cycle have included the isolation and characterization of transposon Tn*5* insertion mutants that show altered growth on minimal media containing DL-3-hydroxybutyrate (DLHB) and/or AA as a sole carbon source (8). To ensure that these mutants had lesions specific to the PHB degradation pathway, we screened for mutants that were unaffected in the ability to utilize acetate and glucose (8). This screening protocol resulted in the initial isolation and characterization of *bdhA* and *acsA2* mutants.

In addition to the mutants that were unable to grow on 3-HB and AA, we also isolated a mutant that exhibited an enhanced growth rate on these carbon sources (8). The Tn*5* insertion in this mutant was found to be closely linked in φM12 transduction to the *acsA2* gene. The Age (for AA growth-enhanced) phenotype was postulated to be due to a regulatory mutation that specifically alters growth on the PHB degradation pathway intermediates 3-HB and AA, since growth on glucose or acetate remained unaffected. In this report, we show that spontaneous mutations that restore growth of the *bdhA* mutant on DLHB minimal medium, without restoration of BDH activity, are tightly linked to the *age* mutation previously reported. We also offer evidence that the apparent suppression phenotype is a consequence of elevated AcsA2 levels, is specific for utiliza-

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TABLE 1. Strains, plasmids, and transposons used in this study

| Strain, plasmid, or transposon | Relevant characteristics[a] | Reference, source, and/or construction |
|---|---|---|
| Strains | | |
| *S. meliloti* | | |
| RCR2011 | SU47 wild type | Rothamsted Experimental Station |
| Rm1021 | SU47 *str-21* | 19 |
| Rm1021 derivatives | | |
| Rm11104 | *age-1*::Tn*5* | 8 |
| Rm11107 | $bdhA_1$::Tn*5* | 8 |
| Rm11143 | *age-1*::Tn*5*-233 | 8 |
| Rm11159 | $bdhA_2$::Tn*5* | 1 |
| Rm11172 | *age-1*::Tn*5*-Tp | This study; Tn*5*-Tp replacement of Tn*5*-233 insertion in Rm11143 |
| Rm11175 | *age-1*::Tn*5*-Tp $bdhA_2$::Tn*5* | This study; φRm11159→Rm11172 |
| Rm11192 | $bdhA_2$::Tn*5 shb-1* | This study; spontaneous growth on DLHB |
| Rm11194 | $bdhA_2$::Tn*5 shb-2* | This study; spontaneous growth on DLHB |
| Rm11196 | $bdhA_2$::Tn*5 shb-3* | This study; spontaneous growth on DLHB |
| Rm11198 | $bdhA_2$::Tn*5 shb-4* | This study; spontaneous growth on DLHB |
| Rm11218 | $bdhA_2$::Tn*5* Ω218::Tn*5*-233 $Hbu^-$ | φTn*5*-233 bank→Rm11192 |
| Rm11219 | $bdhA_2$::Tn*5* Ω219::Tn*5*-233 $Hbu^-$ | φTn*5*-233 bank→Rm11196 |
| Rm11223 | $bdhA_2$::Tn*5* Ω223::Tn*5*-233 $Hbu^-$ | φTn*5*-233 bank→Rm11198 |
| Rm11228 | $bdhA_2$::Tn*5* Ω218::Tn*5*-233 *shb-1* $Hbu^+$ | φRm11218→Rm11192 |
| Rm11229 | $bdhA_2$::Tn*5* Ω219::Tn*5*-233 *shb-3* $Hbu^+$ | φRm11219→Rm11196 |
| Rm11232 | $bdhA_2$::Tn*5* Ω223::Tn*5*-233 *shb-4* $Hbu^+$ | φRm11223→Rm11198 |
| Rm11281 | $bdhA_1$::Tn*5 age-1*::Tn*5*-Tp | φRm11107→Rm11172 |
| Rm11364 | $acsA2_7$::Tn*5* $acsA1_{15}$::Tn*5*-233; deficient in AACS and acetyl-CoA synthetase activities | 20 |
| *E. coli* | | |
| NovaBlue | *endA1 hsdR17* ($r_{K\text{-}12}^-$ $m_{K\text{-}12}^+$) *supE44 thi-1 recA1 gyrA96 relA1 lac*[F′ *proA*$^+$*B*$^+$ *lacI*$^q$*Z*ΔM15::Tn*10*] $Tc^r$ | Novagen |
| BL21(DE3)pLysS | $F^-$ *ompT hsdS*$_B$ ($r_B^-$ $m_B^-$) *gal dcm* (DE3) pLysS ($Cm^r$) | Novagen |
| Plasmids | | |
| pSP329 | IncP cloning vector; $Tc^r$ | 6; S. Porter |
| pGQ105 | pSP329 carrying the *S. meliloti acsA2* gene on a 4.0-kb *Kpn*I fragment | 5 |
| pET30 Xa/LIC | N-terminal His tag expression vector; $Kan^r$ | Novagen |
| pRD112 | pET30 Xa/LIC expressing His-tagged *acsA2* | This study |
| Transposons | | |
| Tn*5* | $Nm^r$ $Sm^r$ | 3 |
| Tn*5*-233 | $Gm^r$-$Sp^r$ | 11 |
| Tn*5*-Tp | $Tp^r$ | 16; S. Klein |

[a] Abbreviations for antibiotic resistance phenotypes: Cm, chloramphenicol; Gm, gentamicin; Kan, kanamycin; Nm, neomycin; Sm, streptomycin; Sp, spectinomycin; Tc, tetracycline; Tp, trimethoprim. Abbreviations for other phenotypes: $Hbu^+$, growth on M9 containing DLHB; $Hbu^-$, no growth on M9 containing DLHB.

tion of LHB but not DHB, and is in fact due to appearance of an L-(+)-3-hydroxybutyryl-CoA synthetase (LHBCS)-dependent pathway for utilization of LHB.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and transposons.** Bacterial strains, plasmids, and transposons used in this study are listed in Table 1. Construction of new strains is described in the text or summarized in the table.

**Media, antibiotics, and culture conditions.** Bacterial cultures in Luria-Bertani (LB), tryptone-yeast extract (TY), and modified M9 minimal medium with different carbon sources and antibiotic concentrations were as previously described (8, 9). Sodium salts of DHB and LHB were purchased from Sigma-Aldrich Chemical Co. (Oakville, Canada). Growth kinetics in liquid medium were determined as described previously (8).

**Genetics and molecular biology.** Bacterial conjugation, φM12 transductions, construction of a Tn*5*-233 random insertion bank in Rm1021, homogenotizations, and transposon replacements were carried out as described previously (10, 12, 13). Standard methods were used for DNA manipulations (2).

**Enzyme assays.** Preparation of cell extracts, protein determination, and assays for BDH activity (NADH formation) and for AACS and acetyl-CoA synthetase activities (acetyl-CoA formation coupled to reduction of $NAD^+$ via malate dehydrogenase and citrate synthase) were carried out as described previously (5, 8). NADH formation was measured using an Ultrospec 2000 spectrophotometer (Amersham Pharmacia Biotech, Baie d'Urfé, Canada). For an assay of AACS activity of purified protein samples, a sonicated cell extract (20 μl of an 11-mg/ml sample) from a culture of TY-grown strain Rm11364 (an *acsA1 acsA2* double mutant) was added as a source of thiolase to convert acetoacetyl-CoA to acetyl-CoA; the amount of acetyl-CoA production was then determined in the assay. LHBCS activity was assayed by coupling the reaction to L-3-hydroxyacyl-CoA dehydrogenase. The reaction mixture (1 ml) contained LHB (varying amounts for kinetic studies with purified protein; 22 μmol for assay of crude extracts), Tris-Cl (200 μmol; pH 8.4), $MgCl_2$ (10 μmol), NAD (10 μmol), CoA (0.2 μmol), L-3-hydroxyacyl-CoA dehydrogenase (8 U, from bovine liver [Sigma-Aldrich]), KCl (100 μmol), ATP (20 μmol), and cell extract or purified protein. The reaction was initiated by addition of ATP. The rate of NADH formation was monitored at 340 nm.

**Overexpression of *acsA2* and purification of AcsA2.** The Novagen (Madison, Wis.) pET30 Xa/LIC (ligation-independent cloning) kit was used. Primers con-

tained vector-compatible overhangs (underlined) and were designed according to the coding sequence of *acsA2* (acsA2licfor, 5′-GGTATTGAGGGTCGCCA AGCAGAACGACCTTTGT-3′; acsA2licrev, 5′-AGAGGAGAGTTAGAGCC AGCCGGCACTACGACA-3′) and synthesized by Sigma-Aldrich. The PCR mixture contained 3 μl of 25 mM $MgCl_2$, 5 μl of 10× *Tli* DNA polymerase reaction buffer (Promega, Madison, Wis.), 1 μl of 10 mM deoxynucleoside triphosphate mix, 2.5 μl of each primer (10 pmol/μl), 2.5 U of *Tli* proofreading polymerase (Promega), 0.2 μg of RCR2011 genomic DNA as the template, and deionized $H_2O$ to make up the final volume to 50 μl. Reactions were carried out in an Eppendorf Mastercycler Gradient thermocycler (Brinkmann Instruments, Mississauga, Canada) using a hot-start amplification protocol (94°C for 2 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 150 s, followed by a final extension at 72°C for 10 min). The single PCR product obtained was purified using the QIAquick PCR purification kit (Qiagen, Mississauga, Canada), followed by extraction with 1 volume of chloroform-isoamyl alcohol (24:1). The purified PCR product was cloned into pET-30 Xa/LIC using T4 DNA polymerase according to the manufacturer's instructions. *Escherichia coli* NovaBlue competent cells were transformed with the reaction mixture, and kanamycin-resistant cells containing the desired recombinant plasmid, pRD112, were thus obtained.

Plasmid pRD112 was transferred into expression strain *E. coli* BL21(DE3) pLysS, and a 5-ml volume of a saturated LB-chloramphenicol-kanamycin culture was used to inoculate a 2.8-liter Fernbach flask containing 500 ml of LB-chloramphenicol-kanamycin. Growth under noninducing conditions was carried out at 37°C with shaking at 200 rpm for 2 h, after which isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, and cells were incubated for another 3 h. Cells were lysed using Bugbuster plus Benzonase (Novagen). Insoluble cell debris was removed by centrifugation, the resulting extract (ca. 8 ml) was applied to a His·Bind resin column (Novagen) with a 1-ml column bed, and 1-ml eluted fractions were collected. Purified protein was visualized as a single band of ca. 72 kDa, and the remaining protein preparation was stored at −70°C in elution buffer containing 50% glycerol.

## RESULTS

**The *age-1* insertion mutation allows the *bdhA* mutant to grow on DLHB without restoration of BDH activity.** In an earlier report (8) we had hypothesized that the Age phenotype of the *age-1* insertion mutant could provide clues to the mechanisms that regulate PHB degradation in *S. meliloti*, since the *age-1* mutant exhibits aberrant growth on the PHB degradation intermediates HB and AA but not on glucose or acetate. To investigate whether the *age-1* insertion mutation confers DLHB utilization on a *bdhA* mutant, we generated a *bdhA age-1* double mutant. The $bdhA_1$::Tn*5* insertion in strain Rm11107 was transduced into strain Rm11172, which bears the *age-1*::Tn*5*-Tp insertion. The resulting double mutant, strain Rm11281, exhibited growth on DLHB that was intermediate between the growth rates of the wild type and the *age-1* mutant (Fig. 1). None of the strains exhibited altered growth on glucose or acetate as a sole carbon source. To investigate the possibility that this phenotypic suppression was mediated by activation of an unidentified, secondary BDH-encoding gene, cell extracts of DLHB-grown Rm11175, a similar *bdhA age-1* double mutant, were assayed for BDH activity (Table 2) by using DLHB as a substrate. The lack of BDH activity in this strain indicated that the suppression of the DLHB growth defect is not due to restoration of BDH activity.

**Isolation of spontaneous mutations that suppress the DLHB growth defect phenotype of the *bdhA* mutant.** By isolating spontaneous mutants that restore the growth of the *bdhA* mutant on DLHB, we investigated whether *S. meliloti* is capable of employing alternative mechanisms for utilization of DLHB. Five independent, TY-grown cultures of Rm11159, carrying the $bdhA_2$::Tn*5* allele, were prepared, and ca. $10^9$ cells from each were spread on M9 DLHB plates. Following incubation at 30°C for 10 days, colonies had appeared on most of the plates. Of the five independent cultures plated, one gave rise to no colonies, even after an extended period of incubation, while the other four cultures gave rise to 1 to 24 colonies each. One colony from each plate was isolated and streak purified twice on DLHB minimal medium; these mutants were designated Rm11192 (*shb-1*), Rm11194 (*shb-2*), Rm11196 (*shb-3*), and Rm11198 (*shb-4*), respectively. The $bdhA_2$::Tn*5* insertion was confirmed to be still present in each spontaneous mutant by transducing the $Nm^r$ marker out of the suppressing background into the Rm1021 wild-type background. The resulting transductants were unable to grow on DLHB, confirming that these strains carried true second-site mutations.

To facilitate further characterization of the spontaneous mutations, a selectable genetic marker was linked to them. A ϕM12 transducing lysate was prepared on a pool of Tn*5*-233 ($Gm^r$-$Sp^r$) insertion mutants. The Tn*5*-233 insertions were transduced into the second-site mutants Rm11192, Rm11196, and Rm11198, and the resulting $Gm^r$-$Sp^r$ transductants were screened by replica plating for inability to grow on DLHB, indicating cotransduction of the Tn*5*-233 insertion with the wild-type allele counterpart of the second-site mutation. Tn*5*-233 insertions linked to the second-site alleles were thus isolated. Strains Rm11218, Rm11219, and Rm11223 each carry a Tn*5*-233 insertion linked to the second-site allele in Rm11192 (*shb-1*), Rm11196 (*shb-3*), and Rm11198 (*shb-4*), respectively.

The cotransduction linkage of the Tn*5*-233 insertions and the second-site alleles was determined by two-factor transduction analysis. In separate backcrosses, each of the linked Tn*5*-233 insertions was transduced into the corresponding original suppressor strain. The resulting $Gm^r$-$Sp^r$ transductants were tested for inability to utilize DLHB. This provided a measurement of the cotransduction frequency of the wild-type counterpart of the suppressor alleles and each linked Tn*5*-233 insertion (Fig. 2). One of the linked Tn*5*-233 insertions, Ω218::Tn*5*-233, was transduced into each of the independently isolated suppressor strains, and similar linkages to each suppressor allele were found, suggesting that all of the suppressor alleles map to the same locus. The Ω218::Tn*5*-233 insertion was also found to have a similar linkage to the *age-1*::Tn*5* insertion, suggesting that the spontaneous suppressor alleles are located near the *age-1* locus on the *S. meliloti* chromosome and that the mechanisms of DLHB suppression in the spontaneous suppressor strains and the *age-1* strain are likely similar. Furthermore, the cell extracts of the suppressor strain Rm11192 did not possess BDH activity (Table 2).

**Plasmid-encoded synthesis of AcsA2 restores growth of the *bdhA* mutant on DLHB.** Since the *age-1*::Tn*5* insertion,which bestows DLHB utilization ability on the *bdhA* mutant (Fig. 1), is tightly linked in transduction to the *acsA2* gene, encoding AcsA2 (8), and also causes elevated expression of the *acsA2* transcript (4), we wished to test whether introduction of *acsA2* on a multicopy plasmid would affect the *bdhA* mutant phenotype. Plasmid pGQ105, carrying *acsA2* as the only predicted functional gene (7, 15) on a 4-kb *Kpn*I fragment, was introduced into the *bdhA* mutant Rm11107, and the transconjugant was tested for the ability to utilize DLHB. Growth kinetics of the wild-type and *bdhA* strains carrying pGQ105 or the vector control plasmid pSP329 confirmed that only strains containing multiple *acsA2* copies exhibit enhanced growth rates on both

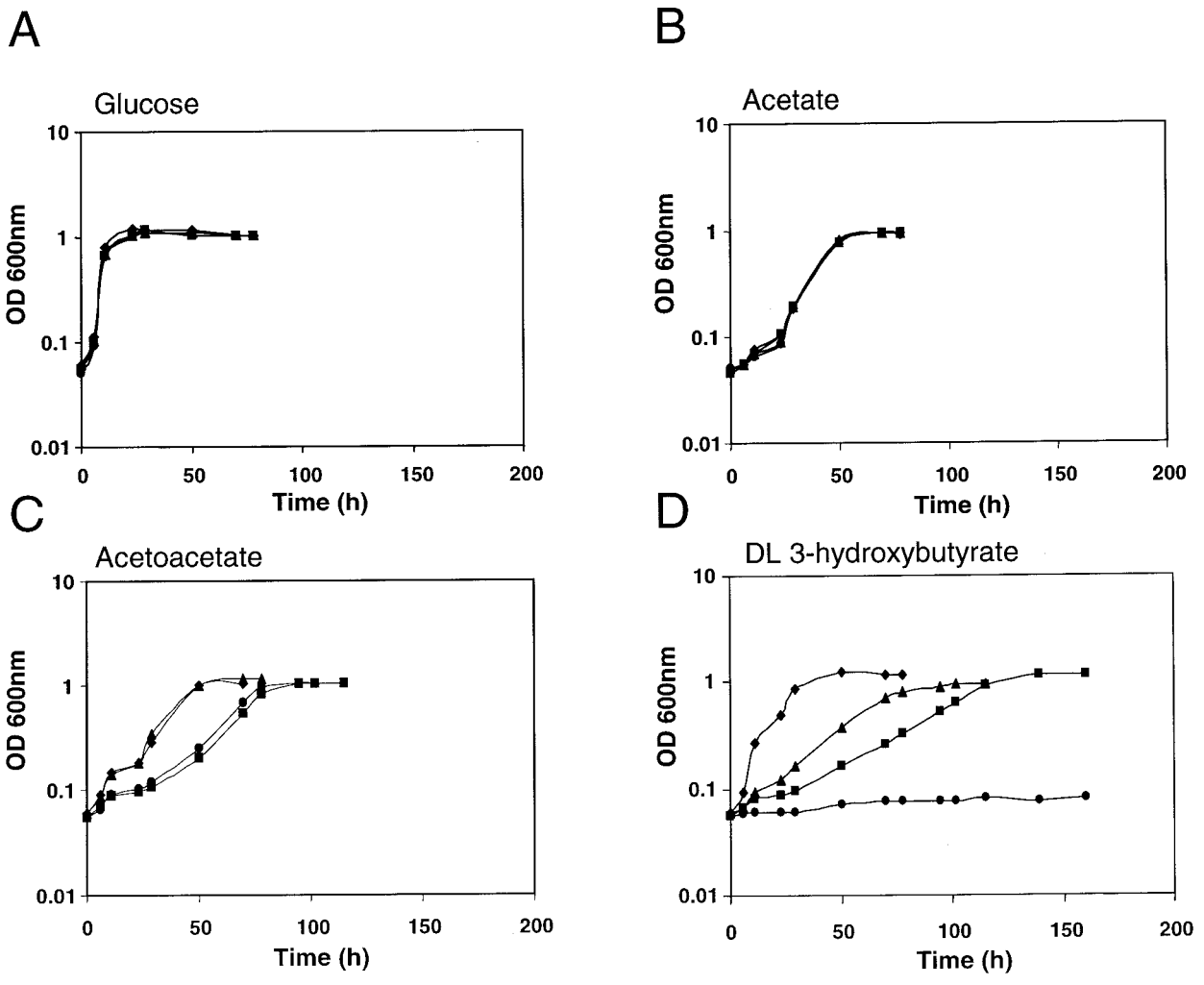


FIG. 1. Growth kinetics of the wild-type strain and representative mutants on different carbon sources. Strains used were Rm1021 (wild type) (■), Rm11172 (*age-1*::Tn*5*-Tp) (◆), Rm11107 (*bdhA*$_1$::Tn*5*) (●), and Rm11281 (*bdhA*$_1$::Tn*5 age-1*::Tn*5*-Tp) (▲). Carbon sources used were 10 mM glucose(A), 30 mM acetate (B), 15 mM AA (C), and 15 mM DLHB (D).

AA and DLHB minimal media (Fig. 3). Although the *bdhA* mutant is unable to grow on DLHB, introduction of the *acsA2* plasmid resulted in a DLHB growth rate considerably higher than that of the wild-type strain that expressed BDH activity. The wild-type strain containing the *acsA2* plasmid had a slightly higher DLHB growth rate, reflecting the presence of BDH activity. On AA, the *acsA2* plasmid enhanced the growth rates of the *bdhA* mutant and the wild-type strains to about the

TABLE 2. Enzyme activities of cell extracts of DLHB-grown cultures[a]

| Strain | Relevant characteristic(s)[b] | Activity (nmol/min/mg of protein)[c] of: | | |
|---|---|---|---|---|
| | | 3-Hydroxybutyrate dehydrogenase | AACS | LHBCS |
| Rm1021 | wt | 44.4 ± 0.8 | 9.8 ± 3.6 | 0 |
| Rm11172 | *age-1*::Tn*5*-Tp | 52.7 ± 2.0 | 27.5 ± 2.2 | 40.7 ± 8.5 |
| Rm11175 | *bdhA*$_2$::Tn5 *age-1*::Tn*5*-Tp | 0 | 42.7 ± 7.3 | 83.3 ± 19.3 |
| Rm11192 | *bdhA*$_2$::Tn*5 shb-1* | 0 | 206.8 ± 23.7 | 98.9 ± 5.2 |
| Rm1021(pSP329) | wt (vector) | 52.0 ± 0.9 | 5.9 ± 1.7 | 0 |
| Rm1021(pGQ105) | wt (*acsA2*) | 42.1 ± 6.8 | 73.9 ± 6.5 | 51.6 ± 19.4 |
| Rm11107(pGQ105) | *bdhA*$_1$::Tn*5* (*acsA2*) | 0 | 115.2 ± 27.5 | 65.2 ± 12.7 |

[a] Cell extracts were prepared from cultures grown in minimal medium supplemented with 15 mM DLHB. Note that *bdhA* mutant controls could not be included because such strains are unable to grow in DLHB.
[b] wt, wild type.
[c] Values are averages from triplicate assays ± standard deviations.

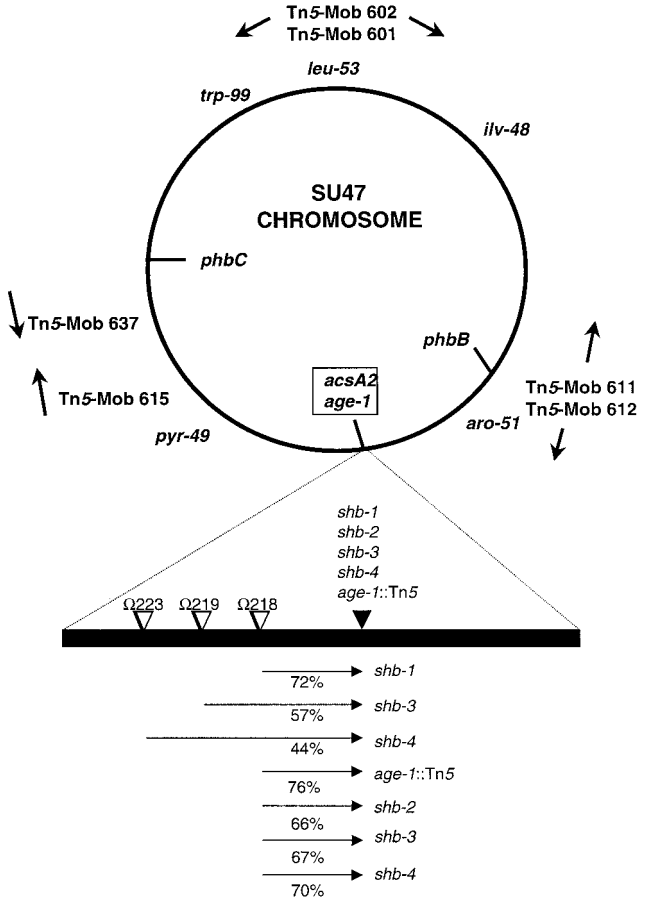


FIG. 2. Genetic map of the *S. meliloti* chromosome, showing the locations of the *acsA2* gene, required for utilization of AA, and *age-1*, an insertion in which results in an increased growth rate on AA and HB. Arrows indicate positions and orientations of Tn*5-mob* insertions. The positions of the auxotrophic markers are from the genetic map of the chromosome (17). The positions of the mapped loci are approximate and are based on conjugation and transduction linkage data (8). The relative order of the Tn*5*-233 insertions and the suppressor alleles depicted is arbitrary. Cotransduction frequencies are given as percentages below the arrows. The tail of each arrow indicates the donor marker and the head represents the recipient in transduction.

same level. Thus, multiple *acsA2* copies not only restore the ability to utilize DLHB to the *bdhA* mutant but also confer on the wild type the enhanced-growth phenotype that is associated with the *age-1* mutation. All strains tested had similar growth rates on glucose and acetate.

**Suppression is specific for utilization of the L(+) isomer of 3-HB.** The observed differences between the extents of enhanced growth of the *age-1* and *bdhA age-1* strains on DLHB point toward the possibility of novel LHB utilization ability conferred by the *age-1* mutation. It seemed reasonable to propose that in the *age-1* mutant, the D(−) and L(+) isomers of HB would be assimilated by BDH-dependent and BDH-independent pathways, respectively. In the absence of BDH activity, however, as in the *bdhA age-1* double mutant, only the L(+) isomer would serve as a potential source of carbon and energy. The growth rate would thus be lower than that of the *age-1* single mutant on DLHB. In order to test this hypothesis, we compared the growth profiles of the wild-type and *bdhA* mutant carrying either the parental vector plasmid pSP329 or the *acsA2* clone pGQ105 on DHB and LHB (Fig. 3). While neither the wild-type nor the *bdhA* mutant control strain was able to grow on LHB, both of these strains had similar growth rates on LHB when they harbored pGQ105. The *bdhA* mutant was unable to utilize DHB even when it carried pGQ105. Therefore, assimilation of LHB in *S. meliloti* occurs only when *acsA2* is overexpressed, and utilization of DHB occurs solely via a BDH-dependent pathway. The suppression of the *bdhA* mutant growth phenotype on DLHB is therefore due to acquisition of the ability to assimilate LHB and does not involve restoration of DHB utilization ability. Interestingly, it was also noted that the growth rate of the wild-type strain Rm1021 on DHB is significantly greater than that observed on DLHB, suggesting that LHB may be an inhibitor of *acsA2* expression or AACS activity.

Ability to grow on LHB upon provision of multiple *acsA2* copies suggested that the AcsA2 enzyme either is directly involved in assimilation of LHB by utilizing LHB as an alternative substrate or may act indirectly by influencing the expression of a latent LHBCS-encoding gene or enzyme activity. To test this, we developed an assay for LHBCS activity based on the coupling of LHB activation to the enzyme L-3-hydroxyacyl-CoA dehydrogenase (see Materials and Methods) and used the assay to measure the levels of LHBCS-specific activity in the mutant and *acsA2*-overexpressing strains (Table 2). While LHBCS was not detectable in the wild-type strain, it was clearly present in extracts from strains that were able to utilize LHB and exhibited enhanced AACS activity. The LHB utilization property is therefore directly related to the introduction of LHBCS enzyme activity resulting from increased expression of *acsA2*.

**Substrate specificity of purified AcsA2 protein.** To test whether the LHBCS enzyme activity detected above was directly due to AcsA2, we carried out enzyme assays with a purified AcsA2 protein preparation. The purified His-tagged AcsA2 protein was active with either AA (i.e., AACS activity) or LHB (i.e., LHBCS activity) as the substrate, but no activity was detected with acetate as the substrate. No AACS activity was detected when the assay was carried out in the absence of the thiolase activity provided by addition of the Rm11364 cell extract. The apparent $K_m$ and $V_{max}$ for AA were 0.30 ± 0.05 mM and 479 ± 12 nmol/min/mg, respectively, compared to 20 ± 3 mM and 259 ± 12 nmol/min/mg for LHB. This further confirms that the LHBCS activity in *S. meliloti* cells that overexpress *acsA2* is directly due to the increased levels of AcsA2.

## DISCUSSION

*S. meliloti* isolate SU47 and its derivatives are naturally unable to use LHB as a sole source of carbon and energy. We have shown, however, that this organism does have the metabolic capacity to utilize LHB. Transposon-generated and spontaneously occurring mutations, as well as plasmid-mediated overproduction of the AcsA2 protein, are able to confer LHB utilization ability. Our growth kinetics and enzyme assay data suggest that *S. meliloti* can use LHB as a growth substrate, but only when the AcsA2 protein is overproduced as a result of genetic alterations. We note that the ratio of AACS activity to LHBCS activity in individual strains varies; for example, strain

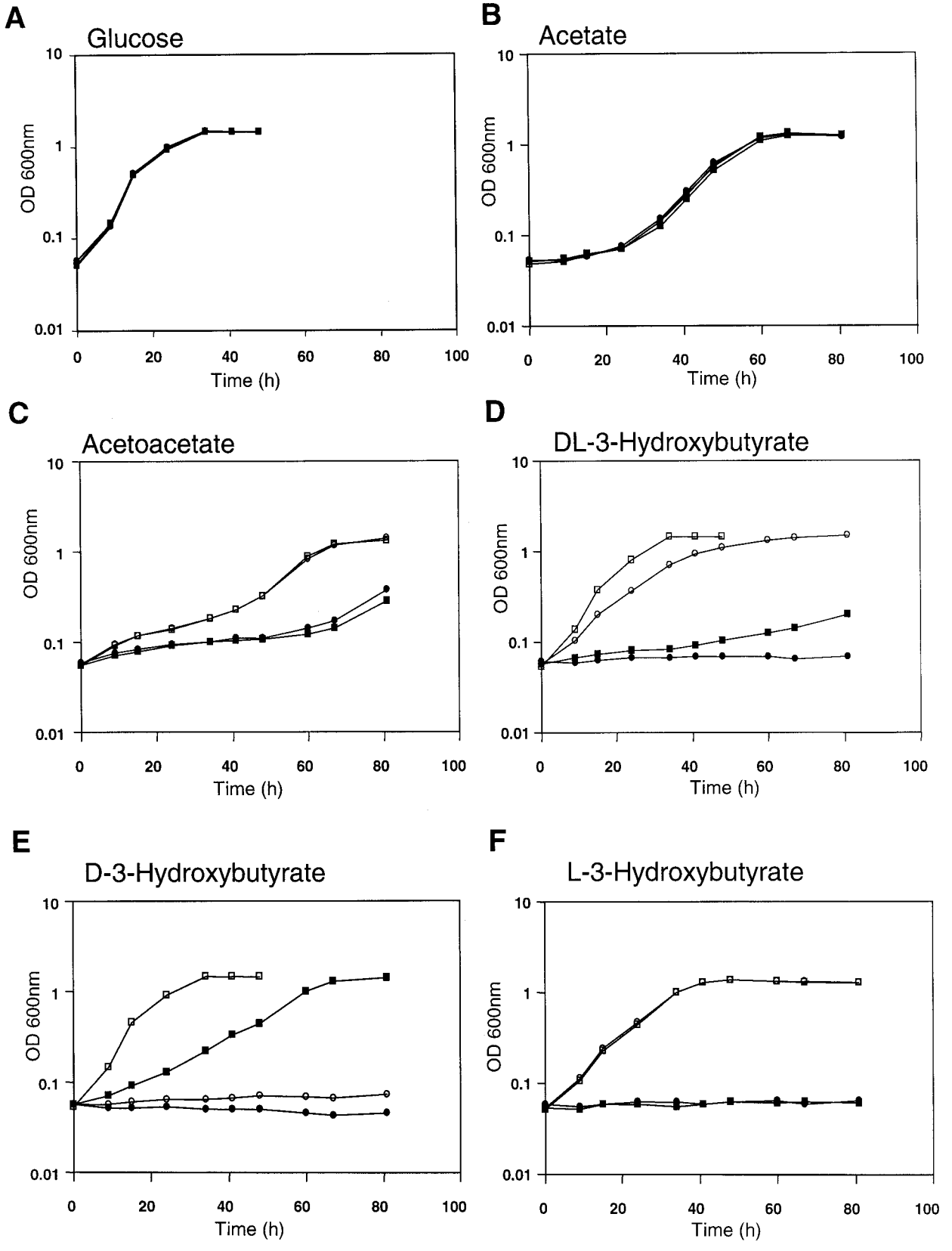


FIG. 3. Growth kinetics of Rm1021 (wild type) and Rm11107 (*bdhA*$_1$::Tn*5*) containing multiple copies of *acsA2* (pGQ105) or parent vector plasmid (pSP329) on different carbon sources. Strains used were Rm1021(pSP329) (■), Rm1021(pGQ105) (□), Rm11107(pSP329) (●), and Rm11107(pGQ105) (○). Carbon sources were 10 mM glucose (A), 30 mM acetate (B), 15 mM AA (C), 15 mM DLHB (D), 15 mM DHB (E), and 15 mM LHB (F).

Rm11192 has an AACS/LHBCS ratio of ca. 2, while strain Rm11175 has an AACS/LHBCS ratio of only ca. 0.5 (see Table 2). This is almost certainly due to differences in the affinities of the two different substrates for AcsA2. We have clearly demonstrated that the purified AcsA2 protein does in fact possess LHBCS activity. The higher $K_m$ and lower $V_{max}$ for LHB than for AA suggest that LHB is not a natural substrate for the enzyme. A similar enzyme with AACS-activating activity was previously isolated from *Zoogloea ramigera* I-16-M, and it is also capable of activating LHB, but with lower efficiency than its activation of AA (14).

There are several possible metabolic fates for LHB following activation to LHB-CoA. It could be further metabolized by 3-hydroxyacyl-CoA dehydrogenase, yielding acetoacetyl-CoA, or by 3-hydroxybutyryl-CoA epimerase, yielding DHB-CoA. Both of these enzyme activities are predicted to be encoded by the *fadB* gene (23) that is present in the *S. meliloti* genome (7, 15). The genome sequence also predicts at least three additional genes encoding 3-hydroxybutyryl-CoA dehydrogenase. Whether either or both of these possible pathways are used during growth on LHB could be tested by mutational analysis of these genes in *S. meliloti*.

Is LHB a naturally utilized carbon source for wild-type *S. meliloti*? We have clearly demonstrated that the organism has the genetic capacity to utilize LHB as a carbon source. Simple up-regulation of the gene expression, without alteration of the coding sequence, was sufficient to confer LHB activation ability on the organism. We have also shown that presence of LHB inhibits the ability to utilize DHB as a carbon source (Fig. 3D and E). The inability of the wild-type cell to utilize LHB is perhaps due to the lack of *acsA2* gene expression induction, or even its inhibition, by exogenous LHB. When the cell is utilizing AA generated from the oxidation of DHB under carbon-limiting conditions, the ability to utilize exogenous LHB could perhaps be deleterious to the maintenance of the PHB degradation process. This might explain why wild-type *S. meliloti* preferentially utilizes DHB over LHB. It is conceivable, however, that under certain environmental and/or physiological conditions, expression of the *acsA2* gene is sufficiently induced by intracellular metabolites and/or by other extracellular environmental signals to result in significant levels of LHB activation.

## ACKNOWLEDGMENTS

This work was supported by a Natural Sciences and Engineering Research Council operating grant to T.C.C.

*Tli* polymerase was a generous gift of VWR Canlab.

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