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Enzymes of Poly-β-Hydroxybutyrate Metabolism in Soybean and Chickpea Bacteroids

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The enzymatic capacity for metabolism of poly-β-hydroxybutyrate (PHB) has been examined in nitrogen-fixing symbioses of soybean (*Glycine max* L.) plants, which may accumulate substantial amounts of PHB, and chickpea (*Cicer arietinum* L.) plants, which contain little or no PHB. In the free-living state, both *Bradyrhizobium japonicum* CB 1809 and *Rhizobium* sp. (*Cicer*) CC 1192, which form nodules on soybean and chickpea plants, respectively, produced substantial amounts of PHB. To obtain information on why chickpea bacteroids do not accumulate PHB, the specific activities of enzymes of PHB metabolism (3-ketothiolase, acetoacetyl-coenzyme A reductase, PHB depolymerase, and 3-hydroxybutyrate dehydrogenase), the tricarboxylic acid cycle (malate dehydrogenase, citrate synthase, and isocitrate dehydrogenase), and related reactions (malic enzyme, pyruvate dehydrogenase, and glutamate:2-oxoglutarate transaminase) were compared in extracts from chickpea and soybean bacteroids and the respective free-living bacteria. Significant differences were noted between soybean and chickpea bacteroids and between the bacteroid and free-living forms of *Rhizobium* sp. (*Cicer*) CC 1192, with respect to the capacity for some of these reactions. It is suggested that a greater potential for oxidizing malate to oxaloacetate in chickpea bacteroids may be a factor that favors the utilization of acetyl-coenzyme A in the tricarboxylic acid cycle over PHB synthesis.

The fixation of atmospheric N_2 by legume-rhizobium symbioses requires the metabolic activities in two organisms to be closely coordinated. The host legume supplies carbon that furnishes the microsymbiont with energy and reductant for the fixation of N_2 and in return receives reduced nitrogen. This carbon is translocated predominantly as sucrose into the root nodules, where it is converted in the host cytosol to organic acids, such as malate, which are the preferred substrates taken up by the bacteroids. Many of the metabolic processes concerned take place in a microaerobic environment which is maintained in the nitrogen-fixing region of nodules to protect O_2-labile nitrogenase (8, 22–24). Regulation of the free O_2 concentration is achieved by a combination of mechanisms, including the maintenance by the bacteroids of a high respiratory demand (5). Although rhizobial bacteroids have the capacity for energy conservation at concentrations of free O_2 that are too low to damage nitrogenase (13), it nevertheless seems likely that availability of O_2 to the bacteroids is a major limiting factor of metabolic processes that support nitrogen fixation.

In many types of symbioses, bacteroids appear to take up more carbon than can be immediately utilized, and under these circumstances, they may form poly-β-hydroxybutyrate (PHB). This polyester storage reserve of carbon and reductant is considered to be an important source of oxidizable substrates to help maintain the respiratory demand of bacteroids and support nitrogen fixation when the supply of photosynthate from the host is reduced, as may occur during extended periods of low light intensity or pod filling (3). However, bacteroids in some symbioses do not accumulate PHB, and moreover, the capacity for symbiotic nitrogen fixation does not appear to be reduced in nodules formed by mutants of *Rhizobium meliloti* and *Rhizobium etli* that are unable to synthesize PHB (6, 18).

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To further our understanding of why this reserve accumulates in some symbioses but not in others, we have examined the enzymatic capacity for PHB metabolism in bacteria that form nodules with soybean (*Glycine max* L.) plants, which may contain more than 50% of their cell mass as PHB (3, 4, 14, 27), and with chickpea (*Cicer arietinum* L.) plants, which have little or no PHB (15). Specifically, we have sought information on whether chickpea bacteroids do not accumulate PHB because they lack the enzymatic capacity or because the enzymes concerned are present but subject to biochemical regulation in situ that inhibits the flux of carbon into PHB.

MATERIALS AND METHODS

Materials. Bacterial cultures were obtained from the Australian Inoculant Research and Control Service, Gosford, New South Wales, Australia, and peat moss cultures of inoculating bacteria were a generous gift from BioCare, Woy Woy, New South Wales, Australia. All enzymes used in coupled assays and other biochemicals were from Boehringer GmbH (Mannheim, Germany) or Sigma Chemical Co. (St. Louis, Mo.).

Growth of plants. Seeds of soybean [*Glycine max* (L.) Merr. cv. Alabaster] and chickpea [*Cicer arietinum* (L.) cv. Tyson] were surface sterilized in 0.8% (wt/vol) NaOCl for 2 min, rinsed thoroughly in running tap water for 12 min, and inoculated with 15 g of *Bradyrhizobium japonicum* CB 1809 and *Rhizobium* sp. (*Cicer*) CC 1192 per 100 g of seed, respectively. Plants were grown in a 3:1 sand-vermiculite mixture in a glass house with average day and night temperatures of 32 and 20°C, respectively, and were supplied weekly with the nitrogen-free nutrient solution of Evans et al. (12) and with tap water as required.

Growth of bacteria. Bacterial cultures were grown for 5 days at 30°C on agar plates made up in modified Bergersen's medium (2, 20) and inoculated into 10 ml of modified Bergersen's medium in loosely capped McCartney bottles. After growth for 5 days at 30°C in an orbital shaker at 200 orbits per min, these cultures were added to 50 ml of the same medium in a 100-ml Erlenmeyer flask stoppered with cotton wool and grown for 3 to 5 days in an orbital shaker until late exponential phase (i.e., A_{660}, ca. 1).

Preparation of bacteroid and bacterial extracts. Bacteroids were isolated from soybean nodules of 41- to 48-day-old plants by the method of Day et al. (9) as follows. Nodules (2 to 6 g) were homogenized with a mortar and pestle in 30 ml of ice-cold medium which contained 50 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), 10 mM KH_2PO_4, 0.4 M mannitol, 2 mM EDTA, 30 mM ascorbic acid, 1% (wt/vol) bovine serum albumin, and 2% (wt/vol) polyvinylpyrrolidone-24. The final pH was adjusted to 7.6 with 0.1 M KOH. The homogenate was filtered through four layers of Miracloth (Calbiochem, San Diego, Calif.), and the filtrate was centrifuged at 4,000 × *g* for 5 min. The pellet

was resuspended in 25 mM TES-NaOH (pH 7.3)–0.2 M NaCl, layered over 30 ml of 70% Percoll in 25 mM TES-NaOH (pH 7.3)–0.2 M NaCl, and centrifuged at 40,000 × *g* for 30 min in a Sorvall SS-34 rotor. The bacteroids were isolated from a band near the bottom of the gradient, diluted fivefold with 25 mM TES-NaOH (pH 7.3)–0.2 M NaCl, and pelleted by centrifuging at 10,000 × *g* for 15 min. Chickpea nodule bacteroids were isolated from 27- to 37-day-old plants by the same method, except that the concentration of Percoll in the gradients was 55%. Free-living cells of CC 1192 and CB 1809 were harvested from liquid cultures by centrifuging at 20,000 × *g* for 15 min, and the pellets were washed by resuspension in 10 ml of 25 mM TES-NaOH (pH 7.3)–0.2 M NaCl.

Bacteroid and bacterial pellets were resuspended in approximately 5 ml of a mixture containing 25 mM TES-NaOH (pH 7.5), 50 mM KCl, 5 mM $MgSO_4$, and 5 mM 1,4-dithiothreitol, sonicated for three 1-min periods at 75% of maximum energy (1,000 W), and centrifuged at 100,000 × *g* for 1 h, and the supernatant was used for enzyme assays. PHB depolymerase activity was measured in pellets from sonicated bacteroid and bacterial suspensions that were centrifuged at 40,000 × *g* for 50 min and resuspended in 9 ml of 50 mM Tris-HCl (pH 8.5).

Analysis of PHB. To determine the PHB content of bacteroids, nodules (2 to 4 g) were crushed with a mortar and pestle in 2 volumes of 50 mM Tris-HCl (pH 8.4), and the homogenate was filtered through four layers of Miracloth and centrifuged at 300 × *g* for 10 min. The supernatant was centrifuged at 5,000 × *g* for 20 min, and the pellet was washed twice with deionized water and dried to a constant weight at 85°C. Pellets of CB 1809 and CC 1192 were washed in approximately 10 ml of deionized water before being dried to a constant weight at 85°C.

PHB analyses were performed by the method of Riis and Mai (19) as follows. Dried bacterial and bacteroid pellets (25 to 45 mg) were incubated with intermittent shaking for 2 h at 100°C in tightly sealed 10-ml vials that contained a mixture of 2 ml of 1,2-dichloroethane, 2 ml of a solution of 4 parts propan-1-ol and 1 part 10 M HCl, and 200 μl of a solution of 2.0 g of benzoic acid in 50 ml of propan-1-ol, which was included as an internal standard. After cooling to room temperature, 4 ml of deionized water was added, the mixture was shaken vigorously for 30 s, and the amount of 3-hydroxybutyrate *n*-propyl ester in the lower phase was determined by gas chromatography at 150 to 160°C with a flame ionization detector and a glass column (2 m by 4 mm) packed with Gas-Chrom Q (80/100) coated with 5% (vol/vol) DC 200 and 5% (vol/vol) QF1 (Alltech, Deerfield, Ill.). Analysis of a known amount of PHB from *Alcaligenes* sp. (Sigma) indicated that the recovery of the 3-hydroxybutyrate *n*-propyl ester was quantitative.

Enzyme assays. Enzyme assays were carried out at 30°C. Unless indicated otherwise, reaction mixtures had a final volume of 1 ml, and activities were calculated from initial rates that were linear. Reaction rates were proportional to the amount of enzyme used, and control mixtures from which the substrate concerned was omitted were used to correct for nonspecific reactions.

Citrate synthase (EC 4.1.3.7) was assayed by monitoring the increase in A_{365} due to the reduction of acetylpyridine-adenine dinucleotide as described by Stitt et al. (21). Reaction mixtures containing 100 mM triethanolamine-HCl (pH 8.5), 3 mM disodium L-malate, 0.22 mM acetylpyridine-adenine dinucleotide, and 20 U of malate dehydrogenase (malate DH) were incubated until equilibrium between malate and oxaloacetate was established, when enzyme extract was added. After the absorbance had reached a steady-state value, the citrate synthase reaction was initiated by the addition of 0.18 mM trilithium acetyl-coenzyme A (trilithium acetyl-CoA). Citrate synthase activity was also assayed by monitoring the increase in A_{412} due to the reaction between 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) and mercaptide ions from CoA by the method of Parvin (17). Reaction mixtures contained 100 mM Tris-HCl (pH 8.0), 0.2 mM oxaloacetate, 0.25 mM DTNB, and 0.1 mM trilithium acetyl-CoA. Both methods gave comparable results, although nonspecific activity was higher with the DTNB method.

Activity of 3-ketothiolase (KT; EC 2.3.1.9) was assayed by monitoring the decrease in A_{303} due to the disappearance of the enolated Mg-acetoacetyl-CoA complex by the method of Karr et al. (14). Reaction mixtures contained 100 mM Tris-HCl (pH 7.8), 25 mM $MgCl_2$, 50 μM acetoacetyl-CoA, and 70 μM CoA (sodium salt). Reactions were initiated with CoA, and an extinction coefficient of 14.21 mM^{-1} cm^{-1} was used to calculate activity.

The remaining enzymes were assayed by monitoring the change in A_{340} due to the oxidation of NAD(P)H or reduction of $NAD(P)^+$. NADH- and NADPH-dependent acetoacetyl-CoA reductase activities (EC 1.1.1.35 and EC 1.1.1.36, respectively) were assayed by monitoring the decrease in A_{340} due to the oxidation of NAD(P)H in reaction mixtures which contained 50 mM *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES)-NaOH (pH 7.6), 0.4 mM NADH or NADPH, 20 mM Mg acetate, and 50 μM acetoacetyl-CoA. Reactions were initiated with acetoacetyl-CoA. NAD- and NADP-dependent malic enzymes (ME; EC 1.1.1.39 and EC 1.1.1.40, respectively) were assayed as described by Copeland et al. (7), except that 50 mM NH_4Cl was included in reaction mixtures. Reactions were initiated with the addition of $MnCl_2$ and L-malate for NAD- and NADP-dependent ME, respectively. Activity of chickpea bacteroid NADP-dependent ME was calculated from the steady-state rate rather than the initial rate. Pyruvate DH (EC 1.2.4.1), isocitrate DH (EC 1.1.1.42), and malate DH (EC 1.1.1.37) were assayed in the reductive direction as described by Copeland et al. (7), and 3-hydroxybutyrate DH (EC 1.1.1.30) was assayed by the method of Wong and Evans (27). Malate DH was also assayed in the oxidative direction in reaction mixtures that contained 50 mM HEPES-NaOH (pH 7.0), 5 mM L-malate, 10 mM L-glutamate, and 1 mM $NAD(P)^+$. The reaction was initiated by the addition of 2 U of glutamate:2-oxoglutarate transaminase (GOT; EC 2.6.1.1). GOT was assayed in reaction mixtures that contained 50 mM HEPES-NaOH (pH 7), 10 mM 2-oxoglutarate, 10 mM DL-aspartate, 0.2 mM NADH, and 5 U of malate DH.

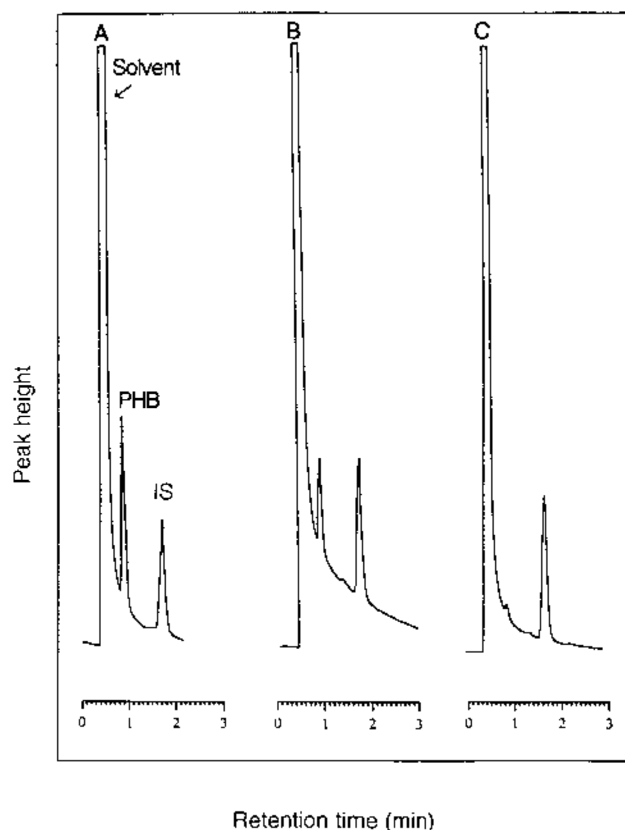

FIG. 1. Analysis of PHB in soybean and chickpea bacteroids. The PHB standard from *Alcaligenes* sp. (A) and extracts from soybean (B) and chickpea (C) bacteroids were subjected to propanolysis, and the products were analyzed as described by gas chromatography. Benzoic acid was used as an internal standard (IS). The large peak with the shortest retention time is the solvent.

PHB depolymerase activity was measured in reaction mixtures which contained 6 ml of 85 mM Tris-HCl (pH 8.5), 4 ml of extract, and additions of PHB as indicated in Table 3. Aliquots (1 ml) were removed at 30-min intervals over a period of 3 h, heated for 3 min in a boiling water bath to stop reactions, and centrifuged at 12,000 × *g* for 10 min. PHB-depolymerizing activity was estimated by measuring the amount of 3-hydroxybutyrate in the supernatant by the method of Williamson et al. (26).

Protein content was determined with Coomassie blue reagent (Bio-Rad, Richmond, Calif.) as described in the manufacturer's instruction, with bovine serum albumin as a standard.

RESULTS

PHB content of bacteroids and free-living forms of the nodulating bacteria. When PHB from *Alcaligenes* sp. was subjected to propanolysis as described above, two peaks were detected in addition to the solvent peak. The peak with the shorter retention time corresponded to the *n*-propyl ester of 3-hydroxybutyrate, and the other peak corresponded to the internal standard (benzoic acid) which was included to monitor the yield of the transesterification reaction (Fig. 1). Similar chromatograms were obtained from the analyses of PHB in bacteroids from soybean and chickpea nodules (Fig. 1) and in the respective free-living bacteria. No additional peaks were observed with mixed samples that contained the bacterial or

TABLE 1. Enzyme activities in soluble extracts from soybean bacteroids and *B. japonicum* CB 1809

Enzyme	Enzyme activity (nmol of product min^{-1} mg of $protein^{-1}$)[a] in:		Soybean bacteroid/ CB 1809 activity ratio
	Soybean bacteroids	CB 1809	
KT	1,739 ± 277 (6)	1,129 ± 262 (5)*	1.5
Acetoacetyl-CoA reductase			
NADH dependent	422 ± 111 (8)	404 ± 85 (5)	1.0
NADPH dependent	48 ± 24 (6)	42 ± 17 (5)	1.1
3-Hydroxybutyrate DH	218 ± 35 (7)	257 ± 77 (7)	0.9
ME			
NAD dependent	44 ± 7 (6)	66 ± 22 (6)	0.7
NADP dependent	33 ± 5 (6)	15 ± 6 (5)*	2.2
Malate DH			
NADH dependent	2,733 ± 150 (3)	2,121 ± 431 (5)*	1.3
NAD dependent	48 ± 4 (3)	83 ± 28 (4)*	0.6
NADPH dependent	1,794 ± 374 (3)	2,205 ± 949 (6)	0.8
Pyruvate DH	25 ± 4 (2)	45 ± 16 (8)*	0.6
Citrate synthase	285 ± 33 (6)	286 ± 51 (6)	1.0
Isocitrate DH	55 ± 20 (6)	91 ± 27 (5)	0.6
GOT	226 ± 26 (3)	359 ± 85 (5)*	0.6

[a] The data (mean ± SE values) were determined from the number of replicate preparations given in parentheses. The extracts from soybean bacteroids and CB 1809 contained 1.1 ± 0.2 (17 replicates) and 1.4 ± 0.3 (15 replicates) mg of protein, respectively. *, significantly different ($P < 0.05$) compared with soybean bacteroid values, as determined by paired t tests assuming unequal variances.

bacteroid extracts and *Alcaligenes* sp. PHB standard (results not shown).

Bacteroids isolated from nodules of 41- to 48-day-old soybean plants contained 344 ± 70 mg of PHB g (dry weight)$^{-1}$ (mean ± standard error [SE] of eight replicate extracts), whereas the PHB content of bacteroids isolated from nodules of 27- to 37-day-old chickpea plants was 15 ± 2 mg g (dry weight)$^{-1}$ (mean ± SE of six replicates). The PHB content of CB 1809 and CC 1192 grown in liquid culture to late exponential or stationary phase, respectively, was 123 ± 21 (six replicates) and 325 ± 46 (seven replicates) mg g (dry weight)$^{-1}$, respectively. Cultures of CB 1809 produced large amounts of exopolysaccharides, which made harvesting of the bacteria difficult after late exponential phase. There was no evidence of exopolysaccharide formation in cultures of CC 1192.

Enzymatic capacity for PHB synthesis in soybean- and chickpea-nodulating bacteria. Bacteroids isolated from Percoll gradients were intact, as indicated by activity of 3-hydroxybutyrate DH being detected only after the preparations were sonicated. The bacteroids were also essentially free of contaminating enzymes from the host cytosol; unsonicated bacteroid preparations contained less than 5% of the total nodule NADH-dependent malate DH activity, an enzyme which is highly active, mostly in the host cytosol (results not shown). Extracts from both types of bacteroids and the corresponding free-living bacteria had similar total protein contents, although the final volume of extracts prepared from CB 1809 was usually three to five times that from CC 1192 because of the difficulty caused by exopolysaccharides in harvesting CB 1809 cells.

Activities of enzymes in the pathway of PHB synthesis (KT and acetoacetyl-CoA reductase) and PHB breakdown (PHB depolymerase and 3-hydroxybutyrate DH), the tricarboxylic acid (TCA) cycle (citrate synthase, isocitrate DH, and malate DH), and related metabolic reactions (NAD-ME, NADP-ME, pyruvate DH, and GOT) were determined in cell extracts from soybean and chickpea bacteroids and from CB 1809 and CC 1192. When the specific activities of these enzymes in extracts from soybean bacteroids and CB 1809 were compared, statistically significant differences were noted for KT, NADP-ME, NADH-dependent malate DH, pyruvate DH, and GOT (Table 1). The bacteroid/free-living bacteria ratios of activities of corresponding enzymes in the two extracts ranged between 0.6 and 2.2. Much greater variability was noted in the comparison between bacteroid and free-living cells of CC 1192. NADH-dependent acetoacetyl-CoA reductase, 3-hydroxybutyrate DH, citrate synthase, and isocitrate DH activities were significantly lower in extracts of chickpea bacteroids than in free-living CC 1192 cells, whereas the bacteroids had higher activities of NADP-ME, NADH-dependent malate DH, and pyruvate DH (Table 2). NAD-dependent activity of malate DH in the direction of malate oxidation was approximately 5% of the corresponding reductive activity in all of the extracts (Tables 1 and 2), whereas NADP-dependent activity was very low (results not shown).

Comparing differences between the species indicated that activities of NADH-dependent acetoacetyl-CoA reductase, and to a lesser extent KT, 3-hydroxybutyrate DH, citrate synthase, and GOT, were much higher in soybean bacteroids than in chickpea bacteroids, whereas NADH- and NADPH-dependent malate DH and NADP-dependent isocitrate DH were more active in extracts of chickpea nodules (Tables 1 and 2). The capacities to form pyruvate in the ME reactions were similar in the bacteroid and free-living forms of CB 1809 and CC 1192, but CB 1809 had a greater capacity than CC 1192 to generate acetyl-CoA from pyruvate. No NAD-dependent isocitrate DH activity was detected in any of the extracts.

PHB-degrading activity in bacteroids and free-living bacteria. When particulate fractions from soybean and chickpea bacteroids and the respective free-living bacteria were incubated in the absence of added PHB, the formation of 3-hydroxybutyrate was detected enzymatically, indicating the presence of PHB-depolymerizing activity (Table 3). The rate of 3-hydroxybutyrate formation was not increased when exogenous PHB from *Alcaligenes* sp. was added to incubation mixtures (Table 3). No 3-hydroxybutyrate was released when PHB was incubated under the reaction conditions in the absence of bacteroid or bacterial extracts.

DISCUSSION

In an earlier microscopic investigation of chickpea nodules, PHB granules were not observed in bacteroids in any of the

TABLE 2. Enzyme activities in soluble extracts from chickpea bacteroids and *Rhizobium* sp. (*Cicer*) CC 1192

Enzyme	Enzyme activity (nmol of product min^{-1} mg of $protein^{-1}$)[a] in:		Chickpea bacteroid/ CC 1192 activity ratio
	Chickpea bacteroids	CC 1192	
KT	470 ± 151 (8)	612 ± 141 (7)	0.8
Acetoacetyl-CoA reductase			
NADH dependent	35 ± 9 (9)	101 ± 19 (5)*	0.3
NADPH dependent	107 ± 34 (5)	79 ± 21 (5)	1.4
3-Hydroxybutyrate DH	43 ± 8 (13)	98 ± 16 (5)*	0.4
ME			
NAD dependent	39 ± 5 (7)	50 ± 8 (5)	0.8
NADP dependent	29 ± 3 (7)	12 ± 4 (5)*	2.4
Malate DH			
NADH dependent	4,867 ± 988 (4)	1,368 ± 300 (6)*	3.6
NAD dependent	181 ± 18 (3)	66 ± 19 (7)*	2.7
NADPH dependent	1,387 ± 366 (3)	405 ± 38 (5)	3.4
Pyruvate DH	12 ± 2 (3)	6 ± 2 (6)*	2.0
Citrate synthase	58 ± 15 (6)	141 ± 28 (6)*	0.4
Isocitrate DH	113 ± 15 (10)	279 ± 56 (6)*	0.4
GOT	113 ± 8 (3)	146 ± 39 (5)	0.8

[a] The data (mean ± SE values) were determined from the number of replicate preparations given in parentheses. The extracts from chickpea bacteroids and *Rhizobium* sp. (*Cicer*) CC 1192 contained 1.6 ± 0.4 (22 replicates) and 1.3 ± 0.5 (18 replicates) mg of protein, respectively. *, significantly different ($P < 0.05$) compared with chickpea bacteroid values, as determined by paired *t* tests assuming unequal variances.

sections examined (15). The present study has confirmed by gas chromatographic analysis that PHB is present in chickpea bacteroids in only a very small amount which, presumably, is insufficient to allow the formation of granules that are visible under the electron microscope. However, free-living cultures of the bacteria that were used to nodulate the chickpea plants accumulated large amounts of PHB, indicating that in this form the bacteria expressed the full complement of enzymes required for PHB synthesis. As expected, substantial quantities of PHB were present in soybean bacteroids and free-living cultures of the nodulating bacteria.

On incubation, particulate fractions from soybean and chickpea bacteroids and free-living cultures of CB 1809 and CC 1192 released 3-hydroxybutyrate, which indicated that these extracts were able to mobilize endogenous reserves of PHB. The rate of 3-hydroxybutyrate release from chickpea bacteroid extracts was substantially lower than that from the other extracts, and this may have been due to the much smaller PHB content. Nevertheless, it seems that chickpea bacteroids retain some capacity for PHB breakdown. The observation that 3-hydroxybutyrate formation did not increase when PHB from *Alcaligenes* sp. was added to the extracts indicates that exogenous PHB was not a good substrate for rhizobial depolymerizing enzymes and is consistent with the suggestion that protein factors associated with PHB granules may be required to facilitate breakdown (1). PHB-depolymerizing activity has been detected in soybean bacteroids, but the enzymes concerned were not characterized (27).

In seeking to identify steps that may be important in directing carbon towards PHB synthesis, we have looked for enzymes that have substantially different specific activities in extracts from chickpea and soybean bacteroids and the respective free-living bacteria. Since such comparisons may reflect enzymatic capacity rather than in vivo activities, only differences that are sufficiently large (i.e., at least two- to threefold) may be considered to have physiological significance. On this basis, it is suggested that extracts from bacteroid and free-living cells of CB 1809 did not differ greatly in their capacity for the enzymes measured, whereas some differences that may have physiological significance were noted between the two forms of CC 1192.

Malate taken up by bacteroids may be decarboxylated to pyruvate by ME or oxidized by malate DH and in this way can provide both acetyl-CoA and oxaloacetate for the TCA cycle. Extracts from chickpea bacteroids contained both NAD- and NADP-dependent ME activities and in this regard were similar to soybean bacteroids, which have two ME, one specific for $NADP^+$ and with a high affinity for malate and the other an NAD-dependent enzyme with a lower affinity for malate (7). In contrast, *R. meliloti* bacteroids contain an NAD-ME, but NADP-ME activity is very low, although the NADP-dependent enzyme is expressed in free-living cells (10, 11). Activity of malate DH measured in the reductive direction with NADH or NADPH was high in all of the extracts, although chickpea bacteroids appeared to have a much greater capacity for this step than soybean bacteroids and free-living CC 1192 and CB 1809. As has been demonstrated in other studies (7, 25), activity of malate DH was much lower in the direction of malate oxidation, which would be the direction of the reaction when the TCA cycle is operating. NAD-dependent malate DH activity appeared to be closer to the capacity for malate decarboxylation by the ME and to the activity of pyruvate DH and TCA cycle enzymes (reference 7 and this study). The activities

TABLE 3. PHB-degrading activity of soybean and chickpea bacteroids and the respective free-living bacteria

Bacteroids or bacterium	PHB-degrading activity (nmol of 3-hydroxybutyrate released min^{-1} mg of $protein^{-1}$)[a]	
	No added PHB	Added PHB[b]
Soybean bacteroids	3.6 ± 0.1 (3)[c]	3.7 ± 0.1 (3)
Chickpea bacteroids	0.6 ± 0.2 (6)	0.6 ± 0.2 (6)
B. japonicum CB 1809	3.1 ± 2.2 (6)	5.2 ± 2.4 (6)
Rhizobium sp. (*Cicer*) CC 1192	8.7 ± 3.7 (5)	10.2 ± 4.2 (6)

[a] Values are means ± SEs. Extracts from soybean and chickpea bacteroids and from *B. japonicum* CB 1809 and *Rhizobium* sp. (*Cicer*) CC 1192 contained 4.4 ± 0.9 (three replicates), 3.9 ± 1.2 (six replicates), 2.5 ± 0.4 (six replicates), and 1.7 ± 1.1 (six replicates) mg of protein, respectively.

[b] *Alcaligenes* sp. PHB was added at 0.6 mg/ml.

[c] Values in parentheses are the numbers of replicate extracts.

of NADH-dependent malate DH, pyruvate DH, KT, and 3-hydroxybutyrate DH measured in soybean bacteroids in this study were comparable to the maximum activities reported for these enzymes by Karr et al. (14); our values for isocitrate DH were lower than those in the earlier study.

The potential for oxidation of malate to oxaloacetate rather than decarboxylation to pyruvate was greater in chickpea bacteroids than in free-living CC 1192 and in free-living and bacteroid cells of CB 1809. This can be seen by comparing the activity of malate DH in the oxidative direction with the combined activities of NAD- and NADP-dependent ME. The ratio obtained for chickpea bacteroids was 2.6, which contrasts with values of 0.6 for soybean bacteroids and approximately 1 for free-living CB 1809 and CC 1192.

The biosynthesis of PHB is initiated with the condensation of two molecules of acetyl-CoA to form acetoacetyl-CoA. The enzyme concerned, KT, was present in extracts of chickpea bacteroids, but its specific activity was much lower than that in soybean bacteroid extracts. Nevertheless, it seems unlikely that the lower activity of KT in chickpea bacteroids would limit PHB synthesis, since CC 1192 had a similarly reduced capacity for this step, compared with CB 1809. The next reaction in the pathway of PHB synthesis is the reduction of acetoacetyl-CoA to *R*-3-hydroxybutyryl-CoA, which is the substrate of PHB synthase. NADH-dependent acetoacetyl-CoA reductase activity was significantly lower in extracts of chickpea bacteroids than in CC 1192, which in turn had less of this activity than extracts of soybean bacteroids and CB 1809. However, it should be noted that there are two types of enzymes that can reduce acetoacetyl-CoA, one (EC 1.1.1.35) which forms *S*-3-hydroxybutyryl-CoA and is concerned with the β-oxidation of fatty acids and another (EC 1.1.1.36) which gives rise to the Renantiomer of the product. Clearly, it needs to be established whether the activity of one or both reductases is reduced in chickpea bacteroids to determine if this apparent difference between the chickpea bacteroid and free-living forms of CC 1192 significantly limits the capacity for PHB synthesis in chickpea nodules.

PHB is thought to accumulate throughout the active nitrogen-fixing period in legume symbioses (3, 14, 27). Although information on fine controls that regulate the partitioning of acetyl-CoA between PHB synthesis and the TCA cycle in bacteroids is limited, the ratio of acetyl-CoA/CoA, the redox poise of the pyridine nucleotide pool, and reduced TCA cycle activity in the microaerobic environment of bacteroids may be important (1, 4, 14). It has been suggested that when soybean bacteroids take up excess malate, an increase in ME activity leads to increases in the acetyl-CoA/CoA and $NAD(P)H/NAD(P)^+$ ratios, which in turn stimulate KT and acetoacetyl-CoA reductase, decrease activity of the TCA cycle DH, and divert carbon to the synthesis of PHB (4, 16). In chickpea bacteroids, the higher capacity for malate oxidation may produce a different balance between malate decarboxylation and oxidation, resulting in a higher steady-state concentration of oxaloacetate that favors the utilization of acetyl-CoA in the TCA cycle rather than for PHB synthesis.

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