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Fermentative and Aerobic Metabolism in *Rhizobium etli*

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Strains of *Rhizobium etli*, *Rhizobium meliloti*, and *Rhizobium tropici* decreased their capacity to grow after successive subcultures in minimal medium, with a pattern characteristic for each species. During the growth of *R. etli* CE 3 in minimal medium (MM), a fermentation-like response was apparent: the O_2 content was reduced and, simultaneously, organic acids and amino acids were excreted and poly-β-hydroxybutyrate (PHB) was accumulated. Some of the organic acids excreted into the medium were tricarboxylic acid (TCA) cycle intermediates, and, concomitantly, the activities of several TCA cycle and auxiliary enzymes decreased substantially or became undetectable. Optimal and sustained growth and a low PHB content were found in *R. etli* CE 3 when it was grown in MM inoculated at a low cell density with O_2 maintained at 20% or with the addition of supplements that have an effect on the supply of substrates for the TCA cycle. In the presence of supplements such as biotin or thiamine, no amino acids were excreted and the organic acids already excreted into the medium were later reutilized. Levels of enzyme activities in cells from supplemented cultures indicated that carbon flux through the TCA cycle was maintained, which did not happen in MM. It is proposed that the fermentative state in *Rhizobium* species is triggered by a cell density signal that results in the regulation of some of the enzymes responsible for the flux of carbon through the TCA cycle and that this in turn determines how much carbon is available for the synthesis and accumulation of PHB. The fermentative state of free-living *Rhizobium* species may be closely related to the metabolism that these bacteria express during symbiosis.

In aerobic bacteria, the tricarboxylic acid (TCA) cycle functions to generate reduced nucleotides by the complete oxidation of pyruvate, which enters the cycle in the form of acetyl coenzyme A (acetyl-CoA). The reduced nucleotides are then used to generate ATP via the electron transport system. Another major function is to produce intermediates for anabolism, and several anaplerotic reactions serve to replenish TCA cycle intermediates which are consumed in these processes (32).

The accumulation of the microbial reserve polyester poly-β-hydroxybutyrate (PHB) in bacteria is well documented (1, 6, 45), as is the presence of PHB in several species of *Rhizobium*, both in the free state (44, 46) and in symbiosis (14, 19, 48). While several different pathways for the production of PHB in various groups of bacteria have been characterized (1), the most common pathway begins with the condensation of two molecules of acetyl-CoA to form acetoacetyl-CoA. Sequential reduction and polymerization reactions produce PHB. This product can be depolymerized and ultimately converted back to acetyl-CoA (Fig. 1). Like the TCA cycle, carbon flux through this pathway is greatly influenced by growth conditions, and the two cycles must compete for a common starting metabolite, acetyl-CoA. However, the function of PHB in cell metabolism in *Rhizobium* species has not been defined.

Studies of *Rhizobium* physiology have been directed mainly towards understanding how this organism participates in symbiosis with leguminous plants, but little is known about how this process relates to the physiology of free-living *Rhizobium* organisms. For instance, it is known that *Bradyrhizobium japonicum* bacteroids accumulate PHB during symbiosis at the same time that they fix N_2 (29) and that bacteroids incubated in low levels of O_2 can utilize PHB as a source of energy and reductive power for N_2 fixation (3). It has also been proposed that PHB accumulation functions as a sink for reductive power and, by sequestering reduced nucleotides, allows the TCA cycle to operate microaerobically (29). Nevertheless, it is not known if free-living *Rhizobium* species operate as microaerophilic organisms that are partially engaged in fermentation.

Previously, we reported that the growth of *Rhizobium etli* CE 3 was impaired when cells were transferred from a rich to a minimal medium (MM) and that this unbalanced growth was associated with a fermentation-like metabolism (12). When other strains or species of *Rhizobium* were tested under the same conditions, a qualitatively similar response was found. In this work, we present the results of physiological studies of this fermentation-like metabolism, which is characterized by the excretion of organic and amino acids into the medium and PHB accumulation. Because PHB, as a sink for reductive power (43), is a fermentative product and because the excretion of organic and amino acids is a documented fermentative response in other bacteria (31), we retain the term "fermentative" to describe the phenomena described here. However, because *Rhizobium* species are strict aerobes, the fermentative response we describe for this genus most probably differs in some respects from that exhibited by, for example, facultative anaerobes (31). *Rhizobium* species are well adapted to live microaerophilically, at least during symbiosis, although it has not been explored whether they have the natural capacity to engage in a fermentative metabolism, as defined above, under low-oxygen conditions in vitro. Significantly, we show that the supplementation of MM with compounds that affect substrate concentrations and/or enzyme activities within or auxiliary to

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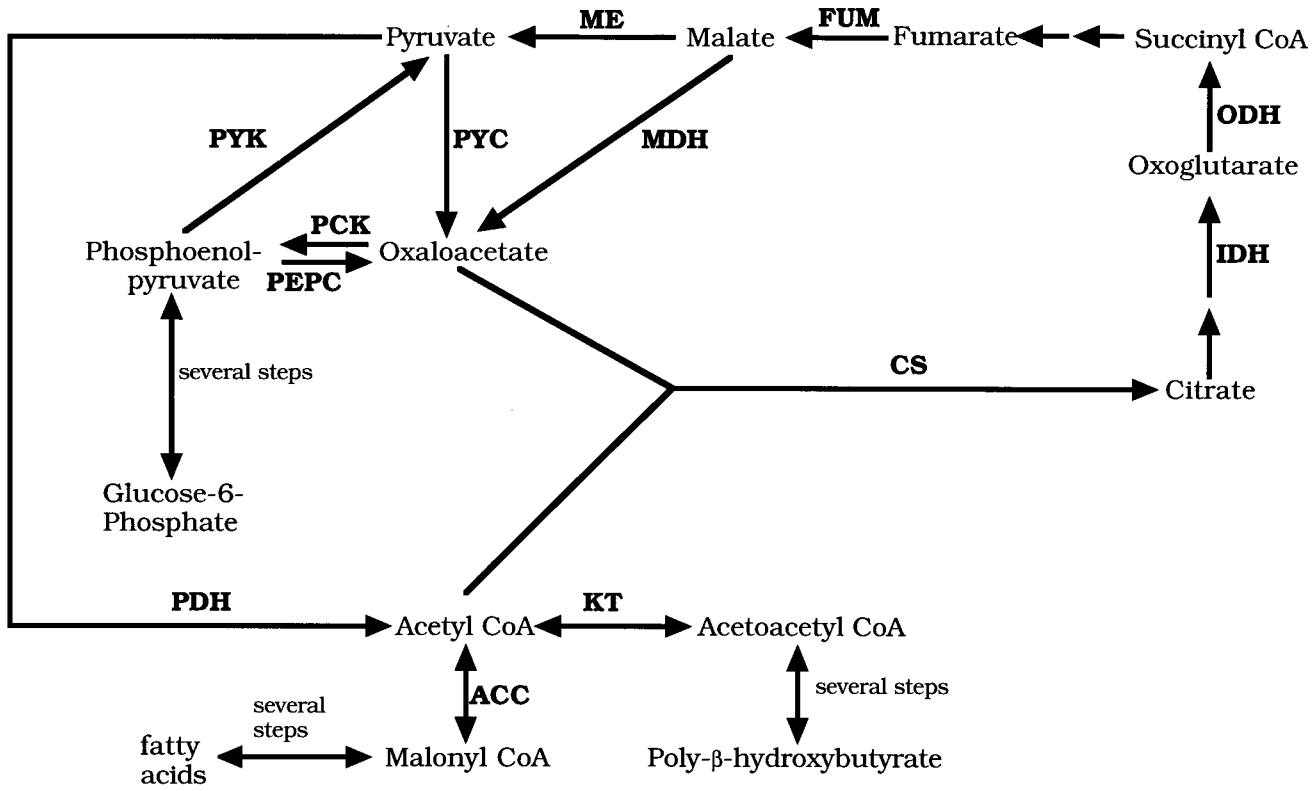

FIG. 1. Scheme of possible enzymatic reactions occurring in *R. etli* and *R. tropici* during growth in MM. Reactions are based on the data in Table 2, although not all potential metabolic interconversions are shown.

the TCA cycle prevents the fermentative response. The results obtained from the experiments using these supplements allow us to present a model to explain why these compounds prevent a fermentative response and stimulate an aerobic metabolism.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used are listed in Table 1. Batch cultures of *Rhizobium* species were grown in 250-ml Erlenmeyer flasks containing 150 ml of medium at 30°C with shaking at 200 rpm. The inoculum used for culturing in MM (which contains 1.26 mM K_2HPO_4, 0.83 mM $MgSO_4$, 0.0184 mM $FeCl_3 \cdot 6H_2O$ [filter sterilized], and 1.49 mM $CaCl_2 \cdot 2H_2O$ [autoclaved separately and added at the end]) (4) was bacteria grown overnight in PY rich medium (5), washed twice with water at room temperature with centrifugations at 15,300 × *g* for 10 min, and diluted to give an initial cell density of 0.05 optical density at 540 nm (OD_{540}) units when added to the MM. For successive subculturing in MM, 24-h MM cultures were harvested and cells were washed, diluted, and added to fresh MM as described above. These cultures were grown for the same period, and the subculturing procedure was repeated several times. MM contained nitrogen (NH_4Cl) and carbon (succinate) sources at final concentrations of 10 mM unless otherwise indicated. Growth in controlled O_2 atmospheres was accomplished by growing cells in batch cultures for 6 h and then transferring 60 ml of the culture to a 160-ml bottle which was then tightly covered with a rubber septum. Air was removed under a vacuum, argon (99% purity) was injected into the bottle and removed under a vacuum, and, finally, different concentrations of O_2 and argon were injected into each bottle. Following 2 h of incubation under the conditions described above, the entire culture was processed for PHB determination. Growth was quantified by measuring the OD_{540} and by determining the protein concentration of the cultures by the method of Lowry et al. (24). At the end of the subcultures, the bacteria were checked to verify that they were free of contamination or of mutations that could alter their growth in MM.

Cell extract preparation and enzyme assays. MM batch cultures were inoculated, maintained, and subcultured as described above. For enzyme assays, cells were harvested by centrifugation at 9,800 × *g* for 10 min at 4°C, washed once in chilled MM, and resuspended to an OD_{540} of 1.0. Twenty-five milliliters of this suspension was centrifuged, and the pellet was resuspended in 5 ml of cell lysis buffer as described previously (19), except that dithiothreitol was omitted when cell lysates were to be assayed for citrate synthase activity. The cell suspensions were lysed by sonication (Soniprep 150, MSE [Dalon Scientific Ltd., London, United Kingdom], equipped with a 1-cm-diameter probe and set at 12-μm amplitude) in an ice-water bath using three 30-s pulses interrupted by 1-min cooling periods. The cell lysates were centrifuged at 12,100 × *g*, and the supernatants were used immediately for enzyme assays. Protein concentrations were determined by a modified Lowry assay (25) using bovine serum albumin as a standard.

Acetyl-CoA carboxylase (ACC) (EC 6.4.1.2) carboxyltransferase activity was determined spectrophotometrically as described elsewhere (15). Citrate synthase (CS) (EC 4.1.3.7) activity was measured by the 5,5′-dithiobis(2-nitrobenzoate) reduction assay of Kurz and LaRue (22). Isocitrate dehydrogenase (IDH) (EC 1.1.1.42), β-ketothiolase (KT) (EC 2.3.1.9), fumarase (FUM) (EC 4.2.1.2), malate dehydrogenase (MDH) (EC 1.1.1.37), and pyruvate dehydrogenase (PDH) (EC 1.2.2.2) activities were determined as described by Karr et al. (19). Oxoglutarate dehydrogenase (ODH) (EC 1.2.4.2) activity was measured by an NAD^+ reduction assay as described previously (18) except that NAD^+ was used in place of acetyl-NAD. Pyruvate carboxylase (PYC) (EC 6.4.1.1) and phosphoenolpyruvate carboxylase (PEPC) (EC 4.1.1.31) activities were measured by MDH-linked assays (2, 33). Phosphoenolpyruvate carboxykinase (PCK) (EC 4.1.1.49) was measured as described by Hansen et al. (16), except that glutathione and NADH were present in the reaction mixture at final concentrations of 1 and 0.2 mM, respectively, and MDH was present at a final concentration of 0.5 U/ml. Pyruvate kinase (PYK) (EC 2.7.1.40) activity was determined by using a lactate dehydrogenase-linked assay (33). Cell lysates and components of the reaction mixture were preincubated for 2 min prior to the addition of substrate, which was used to start the reaction. Cell lysate samples were assayed in triplicate, with one of each set of samples lacking substrate to measure background activity. All assays were done at room temperature. Malic enzyme (ME) activities were measured by using, in separate assays, NAD^+ (NAD^+-ME) (EC 1.1.1.39) and $NADP^+$ ($NADP^+$-ME) (EC 1.1.1.40) as cofactors. The reaction mixtures were incubated at 30°C for 10 min. Complete reaction mixtures inactivated at zero time served as blanks (9).

Determination of organic acids and amino acids. Cell and medium samples (150 and 20 ml, respectively) were withdrawn from cultures grown in MM with and without supplements, and cells were prepared as described previously (5). The medium samples were concentrated by lyophilization, resuspended in 1 ml of 1.25 mM $HClO_4$ containing an appropriate internal standard, and centrifuged at 16,000 × *g* for 10 min at 4°C. The supernatants were filtered through membrane filters (type HA, 0.45-μm pore size; Millipore Corp., Mildford, Mass.) and

TABLE 1. Bacterial strains used in this work

Strain	Original host plant	Geographic origin	Source or reference
R. etli			
CE 3	*Phaseolus vulgaris*	Mexico	5
Viking I	*P. vulgaris*	United States	37
CFN 17	*P. vulgaris*	Mexico	36
Nitragin 8251	*P. vulgaris*	United States	26
CFN 3	*P. vulgaris*	Mexico	36
Bra 5	*P. vulgaris*	Brazil	36
F6	*P. vulgaris*	Mexico	36
R. tropici			
Subgroup A			
CFN 299	*P. vulgaris*	Brazil	27
C-05-I	*P. vulgaris*	Brazil	36
BR 10042	*P. vulgaris*	Brazil	28
BR 845	*L. leucocephala*	Brazil	28
BR 846	*L. leucocephala*	Brazil	28
Subgroup B			
CIAT 899	*P. vulgaris*	Colombia	47
C-05 II	*P. vulgaris*	Brazil	36
BR 863	*L. leucocephala*	Brazil	28
BR 853	*L. leucocephala*	Brazil	28
BR 857	*L. leucocephala*	Brazil	28
R. meliloti			
RCR 2011	*M. sativa*	Australia	39
1021	*M. sativa*	Australia	7
R. me 2	*M. sativa*	Mexico	10
R. me 8	*M. sativa*	Mexico	E. Martínez[a]

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used for organic acid and amino acid analyses. Organic acids from cell and medium samples were separated and quantified by injecting 10 and 50 μl, respectively, into a Waters model 510 chromatograph (Waters Chromatography Division, Millipore Corp.) equipped with a Waters model 490 programmable multiwavelength detector. The separation was performed on three Shodex Ion-Pak KC-811 (Waters Chromatography Division) columns (300 by 8 mm [inside diameter]) connected in a series. The column temperature was 60°C, and the flow rate was 0.9 ml/min. Organic acids were eluted with 1.25 mM $HClO_4$ and reacted with 0.2 mM bromothymol blue–15 mM Na_2HPO_4–2 mM NaOH buffer (pH 10.5) through the reaction coil and detected at 436 nm by color change.

Amino acids were quantified fluorimetrically as described previously (5) or by reverse-phase chromatography using an *o*-thaldialdehyde (OPA) derivatization precolumn and a Nova-Pak C_{18} column (AUTO.TAG OPA [Waters]; 150 by 3.9 mm [inside diameter]). Amino acid separation by reverse-phase chromatography was performed with a 10 to 40% elution gradient of solvents A and B (solvent A, 0.06 M KH_2PO_4–0.06 M K_2HPO_4 [pH 6.65], adjusted with H_3PO_4; solvent B, 45.4% solvent A–18.2% acetonitrile–18.2% methanol–18.2% isopropanol) at a flow rate of 1 ml/min at 40°C. Amino acids were detected with a fluorescence detector (Waters model 470) set at an attenuation of 128, 338 $nm_{\lambda Ex}$, 425 $nm_{\lambda Em}$.

Determination of PHB. PHB was assayed by the spectrophotometric method of Law and Slepecky (23). The single, symmetrical absorption peak for crotonic acid appearing at 235 nm indicated that other reaction products were not interfering with the assay.

Determination of [^{14}C]PHB. Batch cultures of *Rhizobium* spp. were grown in triplicate, as described above, in MM or MM plus biotin (1 μg/ml). After 7 or 14 h of growth, a 30-min pulse of [1,4-^{14}C]- and [2,3-^{14}C]succinate (40 μCi each) was given. After pulse-labeling, the cultures were centrifuged, washed as described above ("Bacterial strains and growth conditions"), and processed for the determination of PHB (23) with the following modification: in the solubilization step with chloroform, two 10-μl aliquots were taken; one was analyzed by the spectrophotometric method described above (23), and the radioactivity incorporated into the other was determined in a scintillation counter (Beckman LS6000SC System).

Determination of O_2 concentration. Cultures used for dissolved-O_2 measurements were grown as batch cultures as described above in 250-ml Erlenmeyer flasks tightly covered with a three-hole rubber stopper, through which were passed a glass tube to deliver O_2 into the medium, an O_2 electrode (model 5739; Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio), and a cotton-plugged glass tube to act as a pressure release. The O_2 dissolved in the culture medium was monitored by using a model SK ARC meter (Yellow Springs Instrument Co.) and a strip chart recorder. O_2 concentrations are expressed as percent dissolved O_2 in the medium.

Determination of cell viability. Total cell numbers in cultures were determined by light microscopy using a Petroff-Hausser chamber. Serial dilutions prepared from the cultures were plated on PY medium, and colonies were counted after 3 days of incubation at 30°C. Cell viabilities were determined as the number of CFU divided by the total number of cells.

RESULTS

Growth of different *Rhizobium* strains. *R. etli* CE 3, *Rhizobium meliloti* 1021, and *Rhizobium tropici* subgroup A (strain CFN 299) and B (strain CIAT 899) strains were subcultivated every 24 h in MM batch cultures containing succinate as the carbon source and ammonium chloride as the nitrogen source. The growth rate of *R. etli* CE 3 decreased by half in the second subculture, and almost no growth occurred in the third subculture (Fig. 2A). The other *R. etli* strains tested (Table 1) behaved similarly. Furthermore, *R. etli* CE 3 maintained this same pattern of growth when subcultured every 12 h, i.e., with inocula prepared from exponentially growing cells (data not shown). When subcultured every 24 h, *R. meliloti* 1021 stopped growing in the fourth subculture, and the *R. tropici* subgroup B strain (CIAT 899) stopped growing in the fifth subculture (Table 1 and Fig. 2B and C). However, the *R. tropici* subgroup A strain (CFN 299) decreased its growth rate by only half in the fifth subculture (Fig. 2D), and this rate was maintained in subsequent subcultures (data not shown). All of the strains tested (Table 1) grew continually if subcultured in PY complex medium. Cell viability, determined as described in Materials and Methods, was not affected when *R. etli* CE 3 was subcultured in MM.

Inclusion of compounds, such as different carbon sources, including glucose (except fumarate and malate), amino acids (except glutamine), purines, pyrimidines, vitamins (except biotin and thiamine), metals, trace elements, nitrate, or urea, in the MM used to subculture the different *Rhizobium* strains gave growth rate decreases similar to those observed in MM subcultures (Fig. 2). The unbalanced growth of *Rhizobium* organisms in MM was not prevented by variations in pH, temperature, or osmolarity.

Effects of biotin, thiamine, fumarate, and malate on culture growth and PHB accumulation. All of the *Rhizobium* strains studied accumulated PHB when they were successively subcultivated in MM. In comparison with the other strains tested, strains of *R. etli* (Table 1), which stopped growing after the second subculture, accumulated higher levels of PHB after each subculture (Fig. 1). For the *Rhizobium* strains studied, a direct correlation between the unbalanced growth in subsequent subcultures in MM and the accumulation of PHB was established (Fig. 2). It has been reported that *Rhizobium trifolii* does not grow in ammonium or nitrate after being previously grown in MM containing either of these compounds as a nitrogen source (34). However, no data regarding PHB accumulation in this strain are available.

In contrast to other bacteria which accumulate PHB only when growth ceases (35, 42), *Rhizobium* organisms accumulated this polymer during growth in the first and second subcultures (Fig. 2 and data not shown). This unique pattern of PHB accumulation occurred even when subcultures were made every 12 h from exponentially growing cells. The accumulation of PHB was also observed in electron micrographs of the *Rhizobium* species studied, and a positive correlation between cellular PHB content, measured chemically, and the presence of intracellular granules was found (data not shown).

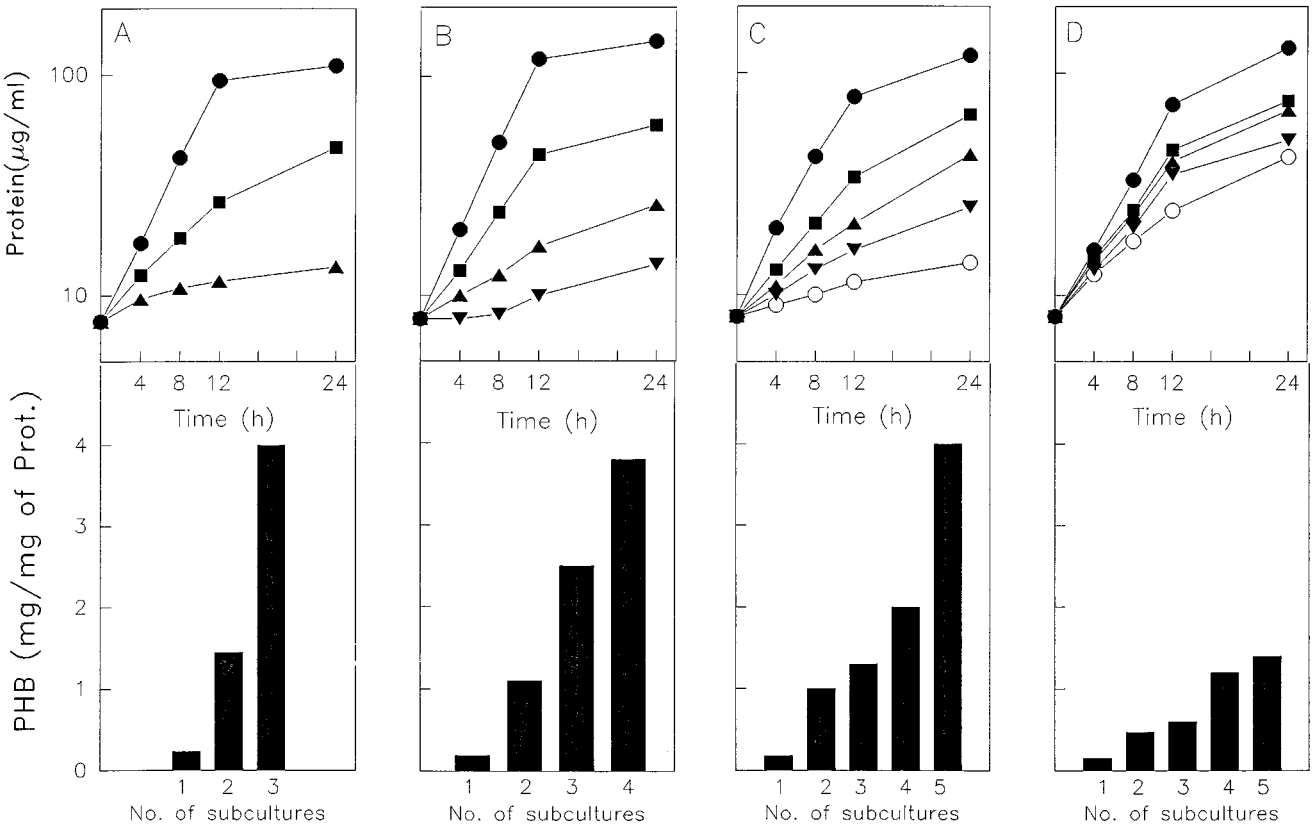

FIG. 2. Growth of and PHB accumulation in different *Rhizobium* strains successively subcultured in MM. (A) *R. etli* CE 3; (B) *R. meliloti* 2011; (C) *R. tropici* CIAT 899 (subgroup B); and (D) *R. tropici* CFN 299 (subgroup A). Growth and PHB accumulation after 24 h of incubation were measured as described in Materials and Methods. Results after the first (●), second (■), third (▲), fourth (▼), and fifth (○) subcultures are shown. Prot., protein.

When biotin, thiamine (Fig. 3), or fumarate and malate (not shown) were added to MM cultures of *R. etli* CE 3, growth was optimal during subculturing and PHB was not accumulated.

PHB turnover. *R. etli* CE 3 cultures grown in MM for 14 h with or without biotin were given a 30-min pulse of [^{14}C]succinate, and the radioactivity incorporated into PHB was measured. The amount of PHB produced was 10-fold higher in the absence of biotin, but the specific radioactivity (counts per minute per milligram of PHB) incorporated into PHB was only 41% of that found in the presence of biotin. When a 30-min pulse was given to cultures grown for only 7 h, the specific radioactivity in PHB was only slightly higher with biotin supplementation than without it. The results of these experiments indicate that PHB is turning over even under conditions in which this polymer is not accumulated, such as in the presence of biotin.

Effects of biotin and thiamine supplementation on enzyme activities. Because *R. etli* CE 3 and *R. tropici* CFN 299 have very different phenotypes when subcultured in MM (Fig. 2A and D), the levels of various enzyme activities in these two strains were determined and are shown in Table 2. With regard to the effect of biotin supplementation on enzyme activities in strain CE 3, four general categories of responses were observed: (i) enzyme activities which were not significantly affected by biotin supplementation in any subculture (ACC, NADP$^+$-ME and NAD$^+$-ME, PEPC, and PYK); (ii) enzymes whose activities were not affected by biotin supplementation in the first subculture but which were maintained, with biotin supplementation, at a significantly higher level of activity in the second and/or third subcultures (CS, IDH, MDH, ODH, and PDH); (iii) enzyme activities which were significantly elevated in biotin-containing cultures in the first and subsequent subcultures (PYC and PCK); and (iv) the enzyme whose activity was substantially decreased over the course of subculturing in MM containing biotin (KT).

Enzyme activities in strain CFN 299 were not affected by biotin supplementation, with the exception of KT, whose activity increased in the presence of biotin in the first and second subcultures. Several enzyme activities were substantially higher in CFN 299 than in CE 3 regardless of biotin supplementation, and they include NAD$^+$-ME, ODH, and PDH. The activity of PYC was severalfold higher in strain CFN 299 than in strain CE 3 grown in MM lacking biotin. Biotin supplementation of strain CE 3 cultures caused an approximately five- to ninefold increase in the activity of the enzyme. PYK was the only enzyme measured whose activity was lower in CFN 299 than in CE 3.

Table 3 presents a comparison of how biotin and thiamine supplementation of strain CE 3 cultures affects the activities of enzymes which require one of these supplements as a cofactor. Compared with nonsupplemented cultures, biotin supplementation increased PYC activity four- to sixfold, while thiamine supplementation resulted in only a twofold increase (Table 3). In thiamine-supplemented cultures, PDH activity was only slightly increased and ODH activity was fourfold higher in the first subculture, but these activities were maintained at this increased level during the second and third subcultures. In nonsupplemented cultures, these activities were undetectable

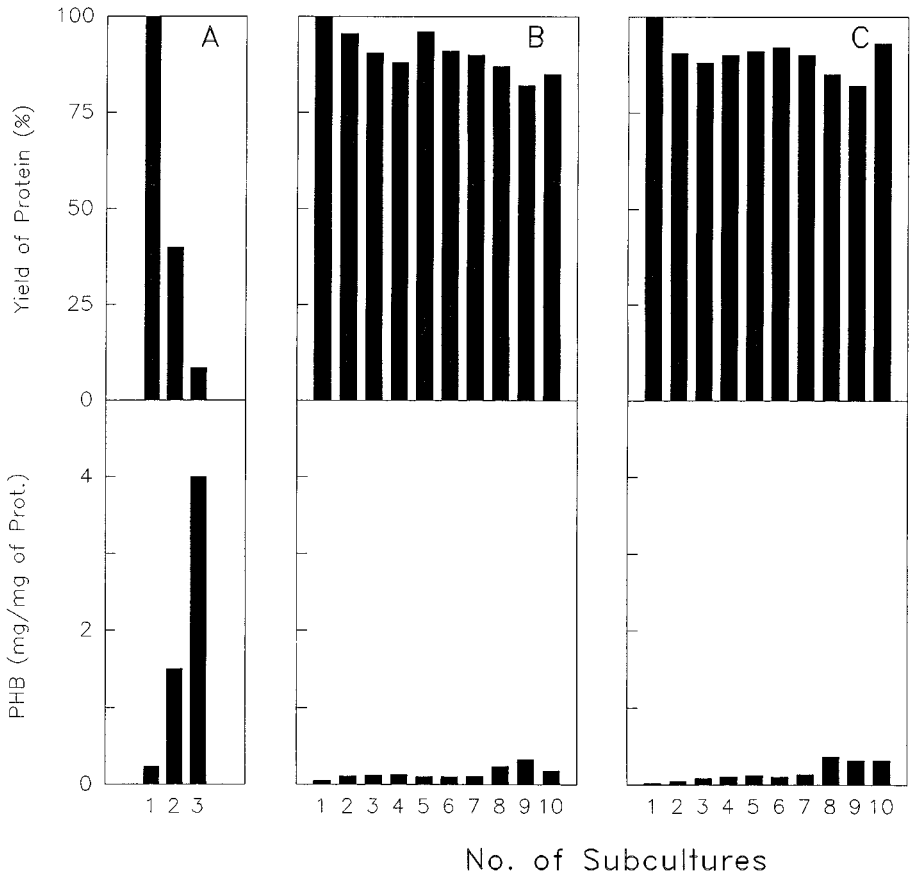

FIG. 3. Growth (yields) and PHB accumulation in *R. etli* CE 3 during successive subcultures in MM alone (A) and supplemented with biotin (1 μg/ml) (B) or thiamine (5 μg/ml) (C). In each experiment, growth and PHB accumulation at 24 h were measured as described in Materials and Methods. The 100% value for yield of protein is from 100 to 110 μg of protein per ml. Prot., protein.

after the first subculture (Tables 2 and 3). Biotin supplementation also maintained the activities of these enzymes following the first subculture (Tables 2 and 3), although the levels of activity were less than those in thiamine-supplemented cultures (Table 3).

Enzyme activities in *R. etli* CE 3 during subculturing in MM supplemented with fumarate and malate. The data presented in Table 4 show that the enzyme activities most affected in cells grown in the presence of malate and fumarate were PDH and PYC (minimum increases of 2.5- and 3-fold, respectively). Enzymes showing a less pronounced increase or maintenance of activity during the course of subculturing were MDH and NAD^+-ME. FUM (Table 4) and $NADP^+$-ME (data not shown) showed no significant increase in the presence of these supplements. It should be noted that for some of the enzymes for which data are presented in Tables 2 through 4, substantial variations in activities were sometimes found between experiments, as noted previously for batch cultures of *Rhizobium leguminosarum* (13). However, there is a difference between Tables 2 and 4 and Table 3, since the sampling was done at 12 and 10 h, respectively. The general trends in enzyme activity changes reported above have been consistent from experiment to experiment.

Excretion of organic acids and amino acids. In addition to PHB, other fermentation products were also produced by *R. etli* CE 3 when it was grown in MM. Organic acids such as 2-oxoglutarate, malate, fumarate, lactate, and γ-hydroxybutyrate were excreted into the medium (Table 5). In contrast, when biotin or thiamine was added to the MM, most of the organic acids, although present in the culture medium at 10 h, were consumed by 24 h in the second subculture, with the exception of pyruvate (Table 5).

Glutamate and, to a lesser extent, alanine were also excreted by *R. etli* CE 3. At the end of the second subculture in MM, the concentrations of these amino acids in the medium were highly elevated. The excretion of these amino acids was drastically reduced in the presence of biotin or thiamine (Table 5). It should be noted that neither the organic acids nor the amino acids were accumulated intracellularly (data not shown).

Effect of O_2 concentration on the growth of *Rhizobium* species. It has been reported that O_2 concentration regulates PHB accumulation in other bacteria (18, 42). PHB production by *R. etli* CE 3 was influenced by O_2 concentration, and PHB was accumulated to different levels during growth in all of the O_2 concentrations tested. When the O_2 concentration was decreased from 100 to 5%, cellular PHB content increased from 0.15 to 0.75 mg/mg of protein, respectively, over the course of 2 h. *R. etli* CE 3 consumed a great deal of dissolved O_2 during the first 12 h of growth, and only by supplying O_2 to the cultures was it possible to maintain a dissolved-O_2 concentration between 18 and 25%. Under these conditions, *R. etli* CE 3 was able to grow well in the second and third subcultures and PHB accumulation was greatly reduced (Fig. 4).

PHB accumulation in the *Rhizobium* strains studied also varied as a result of differences in O_2 consumption and growth rate. In the first subculture, *R. etli* CE 3 had the highest growth

TABLE 2. Enzyme activities in *R. etli* CE 3 and *R. tropici* CFN 299 subcultured in MM in the presence or absence of biotin[a]

Enzyme	Subculture	Enzyme activity in the indicated culture[b]			
		CE 3	CE 3 with biotin	CFN 299	CFN 299 with biotin
ACC	First	20 ± 4	18 ± 3	9 ± 3	8 ± 2
	Second	15 ± 10	26 ± 2	3 ± 5	7 ± 6
	Third	14 ± 1	19 ± 2	10 ± 5	4 ± 2
CS	First	284 ± 34	271 ± 28	227 ± 21	233 ± 26
	Second	248 ± 85	237 ± 32	234 ± 50	243 ± 44
	Third	71 ± 8	255 ± 13	227 ± 18	222 ± 9
IDH	First	372 ± 10	355 ± 21	379 ± 17	385 ± 35
	Second	276 ± 77	339 ± 33	368 ± 58	386 ± 55
	Third	109 ± 7	329 ± 10	346 ± 13	348 ± 26
KT	First	11 ± 13	13 ± 15	NA	90 ± 100
	Second	53 ± 17	6 ± 8	8 ± 6	38 ± 33
	Third	218 ± 34	53 ± 18	NA	NA
MDH	First	10,995 ± 2,406	10,279 ± 1,762	9,356 ± 2,991	10,366 ± 3,081
	Second	9,030 ± 4,753	8,007 ± 484	11,884 ± 4,350	11,021 ± 3,736
	Third	3,509 ± 1,282	10,722 ± 3,207	9,826 ± 2,741	11,732 ± 3,668
NAD^+-ME	First	39 ± 4	36 ± 4	124 ± 18	100 ± 21
	Second	34 ± 6	44 ± 1	137 ± 5	151 ± 6
	Third	32 ± 8	32 ± 5	114 ± 17	104 ± 9
$NADP^+$-ME	First	22 ± 6	18 ± 2	26 ± 5	38 ± 8
	Second	26 ± 1	27 ± 0	28 ± 9	35 ± 11
	Third	22 ± 12	18 ± 6	43 ± 6	41 ± 9
ODH	First	19 ± 5	23 ± 1	40 ± 4	44 ± 2
	Second	9 ± 6	38 ± 1	66 ± 1	66 ± 2
	Third	NA	17 ± 2	61 ± 2	63 ± 4
PCK	First	291 ± 117	936 ± 304	165 ± 5	200 ± 10
	Second	84 ± 18	147 ± 65	227 ± 21	257 ± 5
	Third	NA	349 ± 8	288 ± 22	252 ± 10
PDH	First	14 ± 0	17 ± 2	56 ± 4	56 ± 4
	Second	NA	19 ± 1	41 ± 1	40 ± 3
	Third	NA	8 ± 2	31 ± 1	32 ± 5
PEPC	First	11 ± 1	7 ± 2	16 ± 3	18 ± 2
	Second	10 ± 0	5 ± 3	12 ± 1	11 ± 1
	Third	13 ± 7	8 ± 1	10 ± 3	12 ± 2
PYK	First	86 ± 12	88 ± 2	25 ± 6	26 ± 3
	Second	69 ± 10	94 ± 3	29 ± 2	27 ± 2
	Third	97 ± 1	107 ± 9	37 ± 10	36 ± 1
PYC	First	6 ± 5	53 ± 13	36 ± 1	33 ± 4
	Second	8 ± 3	43 ± 19	33 ± 2	41 ± 3
	Third	5 ± 6	27 ± 4	20 ± 1	30 ± 6

[a] Cells were harvested from duplicate, independently inoculated and maintained cultures at 12 h. Duplicate aliquots of each cell lysate were assayed as described in Materials and Methods.

[b] Results are means ± standard deviations expressed as nanomoles of product formed per minute per milligram of protein. NA, no activity detected.

rate and depleted O_2 from the medium more rapidly than *R. meliloti* 1021, and it also accumulated more PHB. Conversely, *R. tropici* CFN 299 grew at the lowest rate, consumed one-third as much O_2, and had a lower accumulation of PHB (data not shown).

When the standard inoculum used to initiate MM cultures of *R. etli* CE 3 (Fig. 2A) was diluted approximately 16-fold (to give a theoretical initial culture cell density of 0.003 OD_{540} units), O_2 consumption was drastically reduced during growth and the strain grew optimally (to a cell density of approximately 0.10 OD_{540} units in 11 h before transfer). PHB was not accumulated during this incubation time, even after eight subcultures, and PYC, PDH, and ODH activities were similar to those found when supplements such as thiamine were added to MM cultures started at the standard inoculum density (data not shown). Furthermore, when after eight subcultures initiated with low inoculum densities the cells were subcultured at a high inoculum density, the response was the same as that presented in Fig. 2A in regard to growth and PHB accumulation. It should be noted that strain CE 3 completes approxi-

TABLE 3. Enzyme activities in *R. etli* CE 3 during subculturing in MM in the presence or absence of biotin and thiamine

Enzyme	Subculture	Enzyme activity in the indicated culture[a]					
		MM		MM plus biotin		MM plus thiamine	
		10 h	24 h	10 h	24 h	10 h	24 h
PYC	First	12	8	45	48	26	18
	Second	12	9	38	38	17	21
	Third	7	6	30	32	14	15
PDH	First	16	15	20	21	21	41
	Second	NA	NA	13	11	26	34
	Third	NA	NA	17	15	16	33
ODH	First	49	25	82	90	74	114
	Second	26	NA	81	127	112	177
	Third	NA	NA	84	97	95	136

[a] Activities are the averages for duplicate cultures of each treatment harvested at 10 and 24 h, respectively, and are expressed as nanomoles of product formed per minute per milligram of protein. NA, no activity detected.

TABLE 4. Enzyme activities in *R. etli* CE 3 during subculturing in MM in the presence or absence of fumarate and malate[a]

Enzyme	Subculture	Enzyme activity in the indicated culture[b]	
		MM	MM plus fumarate plus malate[c]
FUM	First	833 ± 71	698 ± 1
	Second	486 ± 18	618 ± 1
	Third	531 ± 26	552 ± 15
MDH	First	4,578 ± 548	5,338 ± 38
	Second	3,469 ± 9	7,010 ± 735
	Third	3,638 ± 748	5,969 ± 5
NAD^+-ME	First	93.2 ± 0.7	91 ± 0.8
	Second	88.9 ± 2.2	146.8 ± 3.2
	Third	86 ± 2.9	112.5 ± 6.4
PDH	First	23.5 ± 4.5	57.8 ± 3.8
	Second	4.9 ± 2.3	69.8 ± 9.3
	Third	NA	65.8 ± 4.3
PYC	First	6 ± 0.5	32 ± 5
	Second	5.8 ± 0.5	24.8 ± 0.8
	Third	7.7 ± 0.2	22 ± 2

[a] For subculturing in the presence of fumarate and malate, 100-fold concentrates of fumarate and L-malate were adjusted to pH 6.8, filter sterilized, and added to the MM to a final concentration of 1 mM each.

[b] Activities are averages ± standard deviations for duplicate cultures of each treatment harvested at 12 h and are expressed as nanomoles of product formed per minute per milligram of protein, except for FUM, which is expressed as change in A_{242} per minute per milligram of protein. NA, no activity detected.

[c] Duplicate cultures were independently inoculated and maintained in the presence or absence of 1 mM fumarate and 1 mM malate, as described in Materials and Methods.

mately the same number of generations in 24 h during the first MM subculture regardless of whether the culture is started at a high or low initial cell density.

DISCUSSION

When *R. etli* CE 3 is serially subcultured in MM, a fermentative response (see the introduction) is induced and is characterized by (i) a decrease in the activities of several TCA cycle or auxiliary enzymes, (ii) the synthesis and accumulation of PHB, (iii) the excretion of organic acids and amino acids, and (iv) a reduction in growth rate in each subculture.

The addition of specific supplements to MM or the growth of cultures under conditions in which O_2 was maintained at a relatively high concentration (by sparging or by starting cultures at a low cell density) prevented the fermentative response and allowed an aerobic metabolism to be maintained.

The activities of some of the TCA cycle or auxiliary enzymes measured in strain CE 3 subcultured in MM declined (CS, IDH, and MDH) or disappeared (PCK, PDH, and ODH) by the third subculture, when culture growth was severely restricted (Fig. 2A and Table 2). These results indicate that some TCA cycle activity is impaired under these conditions. On the other hand, the activities of PYK, PEPC, and the MEs (Table 2) did not decrease during subculturing.

In biotin-supplemented MM, strain CE 3 grew well and did not accumulate substantial amounts of PHB during subculturing (Fig. 3B). In addition, the activities of the TCA cycle enzymes were maintained and KT activity was reduced in comparison with the activities in cells grown in nonsupplemented medium. In biotin-supplemented cultures, PYC activity was substantially increased in all subcultures. This enzyme requires a biotin prosthetic group for its activity (20) and appears to be selectively affected by biotin, since the activity of ACC, another biotin-requiring enzyme, was not significantly altered by biotin supplementation (Table 2). While PYC is not normally essential for the growth of bacteria on succinate (41), it is an important anaplerotic enzyme in some species (8, 17). The significantly higher PYC activity present in cells grown with biotin could supply more carbon, in the form of oxaloacetate, to the TCA cycle and allow it to continue operating efficiently. Higher levels of oxaloacetate, the substrate for PCK, may be responsible for maintaining the activity of this enzyme in biotin-supplemented cultures. PCK, which catalyzes the first step of gluconeogenesis, is essential for the growth of *Rhizobium* species in succinate (2). Even though high levels of MDH activity are present in *Rhizobium* species (11, 38) (Tables 2 and 4), some bacteria require PYC activity to provide oxaloacetate in substrate amounts for citrate and phosphoenolpyruvate synthesis. When MDH and PYC are both present, MDH is thought to function mainly in generating energy via the TCA cycle (8).

In MM supplemented with thiamine, strain CE 3 maintained high levels of PDH and ODH activities during subculturing. Because PDH and ODH require thiamine PP_i for activity (32), they may be two of the sites at which thiamine, the precursor of this compound, exerts its effect on growth. In addition, PYC activity was also stimulated by thiamine but to a lesser extent than in the presence of biotin. Conversely, PDH and ODH activities were also maintained with biotin supplementation, but the activities observed with this supplement were lower than those obtained with thiamine. We consider these to be secondary metabolic effects.

Supplementation of MM cultures of strain CE 3 with fumarate and malate substantially increased the activities of PDH and PYC (Table 4) and, along with the slight increase in MDH activity observed, may allow the continued operation of the TCA cycle and gluconeogenesis, as discussed above. In addition, the increased activity of NAD^+-ME in supplemented cultures could supply more pyruvate, the substrate for PYC and PDH. Similar effects on enzyme activities were found when cultures were started at a low inoculum density (to prevent oxygen depletion), indicating that PYC, PDH, and ODH do not require exogenously supplied cofactors or cofactor precursors in order to maintain these high levels of activity (data not shown).

An important component of the fermentative response we

TABLE 5. Excretion of organic acids and amino acids by *R. etli* CE 3 grown in MM with or without vitamin supplements[a]

Culture[b]	Time (h)	μmol of acid/mg of protein[c]								
		Fumarate	Malate	Pyruvate	Lactate	2-Oxog	γ-OHB	Asp	Glu	Ala
MM	10	5.6	10.5	ND	5.0	82.8	0.3	ND	0.5	ND
	24	9.3	20.9	0.9	2.0	12.5	0.3	0.4	2.9	1.1
MM plus biotin	10	3.3	6.8	0.6	5.0	9.8	0.4	ND	ND	ND
	24	ND	ND	0.8	0.3	0.7	ND	ND	ND	ND
MM plus thiamine	10	0.4	3.1	ND	1.4	9.2	0.6	ND	ND	ND
	24	ND	ND	0.2	ND	0.7	0.3	ND	ND	ND

[a] Cells from a second subculture and levels of excreted organic acids and amino acids were determined at the indicated times as described in Materials and Methods. Experiments were repeated several times with similar results, and data from a representative experiment are shown.

[b] MM, MM cultures with 10 mM succinate and 10 mM NH_4Cl; MM plus biotin and MM plus thiamine, MM cultures supplemented with biotin (1 mg/ml) or thiamine (10 mg/ml), respectively.

[c] 2-Oxog, 2-oxoglutarate; γ-OHB, γ-hydroxybutyrate; Asp, aspartic acid; Glu, glutamic acid; Ala, alanine; ND, not detected.

observed was the synthesis and accumulation of PHB, which may function as a carbon reservoir and as a sink for reductive power. The utilization of carbon and NAD(P)H for PHB synthesis may prevent a drastic inhibition of several enzymes of the TCA cycle (11, 18, 29, 30). Strict aerobes such as *Rhizobium* species may be able to maintain a functional TCA cycle even under the oxygen-limited conditions required for symbiotic N_2 fixation.

In contrast to *R. etli* CE 3, *R. tropici* CFN 299 maintained high activities of the enzymes within or auxiliary to the TCA cycle and exhibited only a slight decrease in growth during three subcultures in MM.

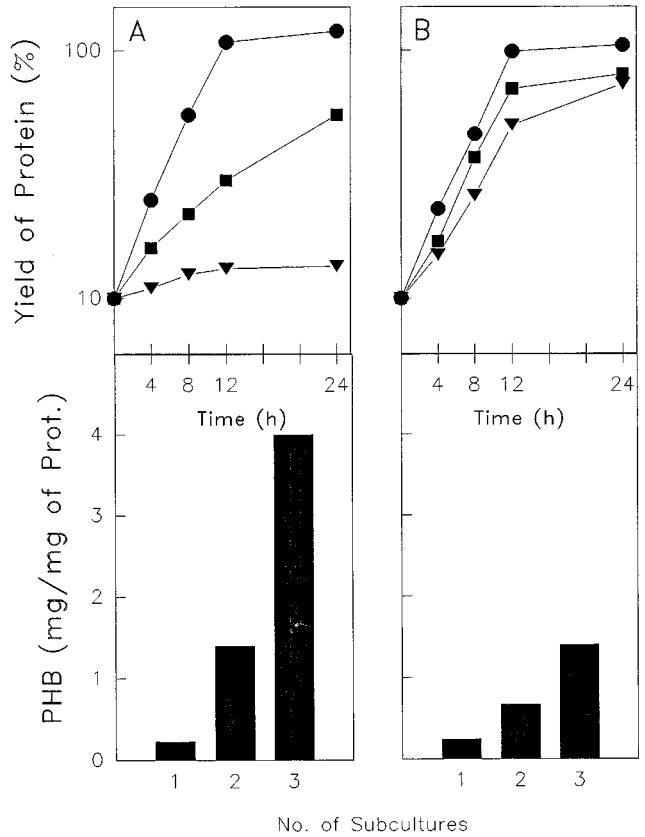

FIG. 4. Effect of dissolved O_2 on growth and PHB accumulation in *R. etli* CE 3. Cells were subcultured in MM, and O_2 concentrations were measured as described in Materials and Methods. At zero time, the O_2 concentration was 20%. (A) No O_2 sparging; (B) O_2 maintained at 18 to 25% with sparging. Results for the first (●), second (■), and third (▼) subcultures are shown. The 100% value for yield of protein is from 100 to 110 μg of protein per ml. Prot., protein.

In summary, *R. etli* must be provided with an adequate supply of biotin or thiamine or substrates for the TCA cycle, including O_2. These treatments result in the maintenance of PYC, PDH, and ODH activities and thereby allow an adequate flow of carbon into the TCA cycle, which is required for sustained culture growth. We believe that the starting signal for the fermentative response may be an increase in cell density and/or a decrease in O_2 concentration (see Results) (unpublished data). The substantially reduced activities of PYC and PDH during the fermentative response would limit the coordinated production of oxaloacetate and acetyl-CoA. The reduced production of these two TCA cycle substrates may account for the general decrease in TCA cycle activity we observed and could also increase the amount of carbon available for PHB synthesis. We have yet to define the mechanism(s) by which oxidizable substrates are excreted by strain CE 3 during fermentative growth. Because this excretion did not result from the accumulation of high intracellular levels of these compounds (data not shown), it bears a resemblance to the specific transport mechanisms operating in fermentative bacteria (21). The fermentative and aerobic states described here are the result of a change in the regulation of the carbon metabolic traffic. A recent report described the role of regulatory proteins in directing the central metabolic traffic of carbon during stress (40), and this regulation in one case involves the phosphorylation of a TCA cycle enzyme (40).

R. etli appears to be well adapted to survive under oxygen-limited conditions in the free state. The fermentative metabolism that occurs under these conditions may be related to that which may occur in symbiosis, in which PHB is synthesized in a microaerobic environment.

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