NOTES

Effect of *aniA* (Carbon Flux Regulator) and *phaC* (Poly- β -Hydroxybutyrate Synthase) Mutations on Pyruvate Metabolism in *Rhizobium etli*

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The *Rhizobium etli* poly- β -hydroxybutyrate synthase (PhaC) mutant SAM100 grows poorly with pyruvate as the carbon source. The inactivation of *aniA*, encoding a global carbon flux regulator, in SAM100 restores growth of the resulting double mutant (VEM58) on pyruvate. Pyruvate carboxylase (PYC) activity, *pyc* gene transcription, and holoenzyme content, which were low in SAM100, were restored in strain VEM58. The genetically engineered overexpression of PYC in SAM100 also allowed its growth on pyruvate. The possible relation between *AniA*, *pyc* transcription, and reduced-nucleotide levels is discussed.

Poly- β -hydroxybutyrate (PHB) synthesis in rhizobia is an important component of their metabolism (3, 4, 12, 17, 21, 24, 25) and is thought to function in draining excess reducing power and carbon from the tricarboxylic acid (TCA) cycle (8, 20). Relative to the wild type, *Rhizobium etli* PHB synthase (*phaC*) mutants (i) excrete high levels of organic acids into the growth medium and contain significantly higher intracellular concentrations of reduced nucleotides, (ii) accumulate higher levels of glycogen but produce similar levels of exopolysaccharide, (iii) have a drastic alteration in global gene expression, and (iv) exhibit severe growth defects with pyruvate as the sole carbon source (4, 12). The lower pyruvate dehydrogenase (PDH) activity in the *R. etli phaC* mutant SAM100 (4) might not be expected to prevent growth on pyruvate, since *Sinorhizobium meliloti* PDH-deficient and *Azorhizobium caulinodans* PDH-null mutants exhibit some growth on this carbon source (or the related carbon source L-lactate) (19, 23).

Pyruvate enters the tricarboxylic acid cycle via the reactions catalyzed by PDH and pyruvate carboxylase (PYC), which are regulated to coordinate the utilization of pyruvate for anabolism (via the anaplerotic production of oxaloacetate by PYC) and catabolism (via acetyl coenzyme A [acetyl-CoA] production by PDH) (26). Rhizobial PYC-null mutants are unable to grow with pyruvate as the sole carbon source (6, 7, 10).

AniA is a global regulator which directs carbon flow into reserve polymers (PHB, exopolysaccharide, and glycogen) in *R. etli* (12) and *S. meliloti* (22). The inactivation of *aniA* in an *R. etli phaC* mutant (i) significantly reduces organic acid excretion and the intracellular concentration of reduced nucleo-

tides, (ii) reduces glycogen accumulation to near-wild-type levels but increases exopolysaccharide production severalfold, (iii) returns global gene expression to a pattern more closely resembling that of the wild type, and (iv) restores the ability to grow on pyruvate (12). Our aim in performing the enzymatic characterization presented here was to establish a metabolic basis for the very different growth phenotypes of the *R. etli phaC* and *phaC aniA* mutants.

TCA cycle, anaplerotic, and gluconeogenic enzyme activities. *R. etli* wild-type strain CE3 and the mutants SAM100 (CE3 *phaC*:: Ω -Km^r), VEM58 (CE3 *phaC*:: Ω -Sm^r/Sp^r *aniA*::Tn5), and VEM5854 (CE3 *aniA*::Tn5) were described previously (4, 12). Growth conditions and the preparation of rich medium (PY) and biotin-supplemented minimal medium (MM) were described previously (6). Culture growth was determined by reading optical density at 540 nm (OD₅₄₀) (7). Cell extracts were prepared by sonication in sonication buffer lacking KCl (10), and those used for the assay of biotin-dependent carboxylases were dialyzed against sonication buffer before use. PYC (EC 6.4.1.1) activity was measured by a ¹⁴CO₂ incorporation assay (10). Propionyl-CoA carboxylase (PCC; EC 6.4.1.3) activity was determined in reaction mixtures containing the following (final concentrations): Tris-HCl (pH 8.0), 50 mM; MgCl₂, 2.5 mM; bovine serum albumin, 0.5 mg ml⁻¹; dithiothreitol, 1 mM; KCl, 40 mM; ATP, 1 mM; NaH¹⁴CO₃, 7 mM (specific radioactivity, 0.7 μ Ci mmol⁻¹); and propionyl-CoA, 0.5 mM. The incorporation of ¹⁴CO₂ was determined as described previously (10). Aspartate aminotransferase (AAT; EC 2.6.1.1) was assayed as described by Kenealy et al. (16) but with Tris-HCl at pH 8.0. Citrate synthase (CS; EC 4.1.3.7), isocitrate dehydrogenase (IDH; EC 1.1.1.42), malate dehydrogenase (MDH; EC 1.1.1.37), NAD⁺-dependent malic enzyme (NAD-ME; EC 1.1.1.39), PDH (EC 1.2.2.2), oxoglutarate dehydrogenase (ODH; EC 1.2.4.2), phosphoenolpyruvate car-

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TABLE 1. Activities of metabolic enzymes in *R. etli* strains

Enzyme	Enzyme sp act in strain ^a :		
	CE3	SAM100	VEM58
AAT	349 ± 40	257 ± 88 (0.74)	292 ± 111 (0.84)
CS	266 ± 68	106 ± 56 (0.30)	445 ± 92 (1.28)
IDH	333 ± 46	250 ± 17 (0.74)	301 ± 27 (0.90)
MDH	7,575 ± 2,281	9,594 ± 1,317 (1.27)	7,197 ± 1,174 (0.95)
NAD-ME	180 ± 76	145 ± 51 (0.81)	ND
ODH	34 ± 5	23 ± 4 (0.68)	33 ± 8 (0.97)
PCC	12 ± 3	18 ± 2 (1.50)	12 ± 0 (1.00)
PCK	752 ± 245	261 ± 95 (0.35)	673 ± 48 (0.89)
PDH	29 ± 3	12 ± 1 (0.41)	49 ± 5 (1.69)
PYC	85 ± 0	16 ± 5 (0.19)	74 ± 25 (0.87)
PYK	24 ± 5	26 ± 13 (1.08)	34 ± 7 (1.42)

^a Cells were harvested from 16-h MM-pyruvate cultures. Activities are given in nanomoles per minute per milligram of protein. Values are means ± standard errors of the means for two or three experiments. Values in parentheses are normalized to the activities produced by strain CE3, set at unity. ND, not determined.

boxykinase (PCK; EC 4.1.1.49), and pyruvate kinase (PYK; EC 2.7.1.40) were assayed as described previously (11). Protein was assayed by the Bradford method (2).

Metabolic enzymes were assayed in extracts of *R. etli* CE3, SAM100, and VEM58 prepared from cells cultured in MM-pyruvate. Table 1 shows that SAM100 had activities of PDH, PYC, PCK, and CS ranging from 19 to 40% of those of the wild type. The activities of IDH, AAT, NAD-ME, and ODH were less severely diminished in SAM100 and ranged from 68 to 81% of those of the wild type, while PYK, MDH, and PCC activities were not reduced. In contrast to the reduced activities found in SAM100, VEM58 produced near-wild-type levels of PYC, CS, PDH, and PCK.

Because the *R. etli* PYC-null mutant 12-53 has the same succinate-positive pyruvate-negative growth phenotype as the *phaC* mutant SAM100 (6, 7, 12), we determined PYC activity and holoenzyme production in the *R. etli* strains grown under different conditions. Under the growth conditions tested, SAM100 had 62 to 76% less PYC activity than wild-type strain CE3 and the *phaC aniA* mutant VEM58 had a somewhat higher than wild-type level of PYC, while the *aniA* mutant VEM5854 had an activity intermediate between those of CE3 and VEM58 (Table 2). Previously, we showed that, compared to levels in cultures grown in MM-succinate or PY, PYC activity and *pyc* transcription in *R. etli* CE3 were increased in cultures grown in MM-pyruvate (6, 7). In contrast, PYC activity in SAM100 was not significantly different in any of the

TABLE 2. PYC activity in *R. etli* strains

Strain	PYC sp act in extracts of cells cultured in ^a :		
	MM-succinate	MM-pyruvate	PY
CE3 (wild type)	35.2 ± 1.3	62.1 ± 0.9	42.4 ± 7.6
SAM100 (<i>phaC</i>)	13.5 ± 1.1	14.9 ± 0.9	13.1 ± 0.1
VEM58 (<i>phaC aniA</i>)	56.0 ± 0.5	71.8 ± 0.8	50.5 ± 1.5
VEM5854 (<i>aniA</i>)	23.7 ± 0.4	37.6 ± 0.9	25.8 ± 2.5

^a Cells were harvested from 16-h cultures. Specific activity is expressed in nanomoles per minute per milligram of protein, and values are means ± standard errors of the means for duplicate assays performed on two independent cultures.

FIG. 1. Analysis of holo-PYC protein in the *R. etli* wild-type and mutant strains. Cultures were grown in PY, MM-succinate, or MM-pyruvate for 16 h. Fifty micrograms of total protein was run in each lane, and holo-PYC was detected by Western blotting as described in the text. Band intensities (pixels), normalized to wild-type strain CE3, are indicated.

growth media tested (Table 2). PCC (9) activity was similar in all of the strains under a given growth condition (results not shown), indicating that differences in PYC activity were not due to a general inability to produce or biotinylate biotin-dependent enzymes.

PYC activity was also measured in intact cells of mutant SAM100. For these assays, cells from MM-pyruvate cultures were washed twice in buffer containing 100 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, and 100 µg of chloramphenicol per ml and resuspended in fresh buffer to an OD₅₄₀ of 9 to 10. Reaction mixtures (0.5 ml, final volume) contained the following: cell resuspension, 466 µl; NaH¹⁴CO₃, 10 mM (specific radioactivity, 0.95 µCi mmol⁻¹); and pyruvate, 20 mM. Reactions were initiated by the addition of pyruvate, and mixtures were incubated at 30°C with gentle agitation. At intervals, 50-µl aliquots of the mixtures were combined with an equal volume of 2.4 N HCl and prepared for scintillation counting as described previously (10). In the in vivo assays, SAM100 incorporated 58% less CO₂ than CE3, which fixed 2.74 nmol min⁻¹ (mg of protein)⁻¹. The PYC mutant 12-53 (6) was devoid of CO₂-fixing activity, indicating that PYC was responsible for the CO₂ fixation observed in these assays. We conclude that the partial PYC deficiency detected in SAM100 in vitro (Table 2) is of a similar magnitude in vivo.

Quantitation of holo-PYC levels. Proteins in cell extracts were separated in sodium dodecyl sulfate–10% polyacrylamide gels and transferred to polyvinylidene difluoride membranes as described previously (6). Holo-PYC was detected with streptavidin-horseradish peroxidase (6) and quantitated with the National Institutes of Health Image program (version 1.62). The holo-PYC content of all of the strains (Fig. 1) showed a good correlation with the PYC specific activities found in cell extracts (Table 3). Biotin uptake (6), incorporation into total

TABLE 3. Enzyme activities in *R. etli* SAM100 expressing cloned metabolic genes

Enzyme	Activity in SAM100 containing ^a :				
	pLAFR1 (none)	pPC1 (PYC)	pRK7813 (none)	pCcsA (CS)	pMOP5 (PCK)
PYC	14 ± 1	79 ± 7	ND	ND	ND
PDH	18 ± 1	47 ± 13	ND	ND	ND
CS	84 ± 4	62 ± 2	352 ± 9	2,618 ± 213	ND
PCC	16 ± 0.1	11 ± 0.9	ND	ND	ND
PCK	180 ± 37	277 ± 44	215	ND	325

^a Plasmids are described in the text; plasmid-encoded enzymes are in parentheses. Strains were cultured in MM-pyruvate for 16 h, and activities are expressed in nanomoles per minute per milligram of protein. Values are means ± standard errors of the means from two or three independent experiments; values without standard errors were obtained in a single experiment. Growth yields were determined for duplicate cultures by subtracting the initial culture OD₅₄₀ from that obtained at 24 h and are as follows for cultures containing the indicated plasmids (left to right): 0.038, 0.688, 0.031, 0.081, and 0.057. ND, not determined.

protein (5), and distribution among biotin-containing proteins were determined during growth of the strains in MM-succinate containing [³H]biotin (6). All of the strains had similar kinetics of biotin uptake and incorporation into cellular proteins (results not shown). The [³H]biotin content of PYC, PCC (9), and an unidentified 14-kDa biotin-containing protein (6) was quantitated and expressed as the fraction of the total label incorporated in all of the proteins. The only significant difference in the distribution of [³H]biotin among the proteins was that SAM100 contained only 20% as much [³H]biotin in PYC and 1.5-fold more label in the 14-kDa protein relative to the other strains (results not shown).

***pyc* transcription assays.** Plasmids containing the *R. etli pyc* gene fused to a *gusA* (encoding β-glucuronidase [GUS]) reporter gene were described previously (7). GUS activities produced by the *pyc::gusA* gene fusion introduced into strains CE3, SAM100, and VEM58 were determined in cultures grown in MM-pyruvate for 16 h as described previously (7). The SAM100 fusion strain had 53% less GUS activity than the wild type, which produced 3.0 nmol min⁻¹ (mg of protein)⁻¹. Significantly, GUS activity in the VEM58 fusion strain was nearly identical (3.1 nmol min⁻¹ [mg of protein]⁻¹) to that of the wild type. This indicates that the different levels of holo-PYC in these strains are determined at the level of *pyc* transcription. Extract mixing experiments to measure the stability of PYC activity over time at 30°C showed <5% differences in PYC activity in CE3 or SAM100 extracts incubated individually or mixed together (results not shown).

Enzyme activities in SAM100 expressing cloned metabolic genes. In an attempt to overcome the pyruvate growth defect of mutant SAM100, we introduced plasmids encoding selected enzymes which our assays (Table 1) revealed as being significantly lower in the mutant. Plasmids containing genes encoding PYC from *R. etli* (pPC1 [6]), PYC from *S. meliloti* (pTH424 [10]), CS from *Rhizobium tropici* (pCcsA [14]), or PCK from *Rhizobium* sp. strain NGR234 (pMOP5 [18]) were introduced into SAM100 by triparental matings as described previously (6). SAM100 containing pPC1 had 5.4-fold more PYC activity than the control strain, which contained vector pLAFR1 (6) without an insert (Table 3). Strain SAM100/pPC1 had a wild-type growth yield, while the vector control exhibited minimal

growth (Table 3). SAM100 containing *S. meliloti pyc* had 2.4-fold-higher PYC activity than the pRK7813 (10) vector control and attained a growth yield nearly identical to that of SAM100/pPC1 (results not shown). The PYC-overexpressing strain SAM100/pPC1 also had high PDH activity. While it would be of interest to determine the effect of PDH overexpression in SAM100, the unavailability of a plasmid containing all of the genes encoding the subunits of the enzyme prevented such an attempt. SAM100/pCcsA had a 7.4-fold increase in CS specific activity relative to SAM100 containing vector pRK7813, but its growth yield was not increased significantly. SAM100/pMOP5 did not grow on MM-pyruvate, although only a 1.5-fold increase in PCK specific activity was achieved relative to the vector control containing pRK7813 (Table 3).

In this report we show that strain SAM100 has lower PYC activity because it contains less PYC holoenzyme, which appears to result from a lower level of *pyc* transcription and not from a deficiency in biotin uptake or a general reduction in the ability to incorporate biotin into biotin-containing proteins. The severely reduced CS and PDH activities in SAM100 could result from their inhibition (8) by the high level of reduced nucleotides present in this strain (4). Although the *R. etli* PYC is inhibited by NADH (13), the agreement between our in vitro and in vivo PYC assays indicates that this mode of inhibition is not significant in SAM100. Pyruvate excretion by a *Ralstonia eutropha* PHB-negative mutant has been linked to the inhibition of PDH by acetyl-CoA (15). We hypothesize that the high PYC activity in SAM100/pPC1 provides more oxaloacetate for the CS reaction, thus lowering the concentration of acetyl-CoA and preventing the inhibition of PDH. This may explain why high PDH activity is found in SAM100 overexpressing PYC. SAM100 was also able to grow in MM-pyruvate supplemented with L-aspartate (results not shown), which is converted to oxaloacetate via AAT (Table 1) and so eliminates the requirement for PYC (6, 7).

The inactivation of *aniA* in SAM100 restored *pyc* transcription and holo-PYC levels to those of the wild type, suggesting a possible regulatory role for AniA. The transcriptional control of metabolic genes by reduced nucleotides and other redox signals has been amply demonstrated in bacteria (1). It is reasonable to suggest that the high levels of reduced nucleotides found in SAM100 could be responsible for its highly altered pattern of global gene expression (12). As a working hypothesis, we suggest that *pyc* transcription could be controlled by a redox signal cascade in which AniA participates. For example, the significantly higher level of exopolysaccharide synthesis in the *phaC aniA* mutant VEM58 could maintain the intracellular concentration of reduced nucleotides in this strain at wild-type levels (12), since the gluconeogenic biosynthesis of exopolysaccharide precursors from pyruvate would consume substantial quantities of NADH (26). While consumption of reduced nucleotides would also occur in the synthesis of glycogen precursors, the relatively modest increase in glycogen synthesis in *phaC* mutant SAM100 (4) may be insufficient to lower the concentration of reduced nucleotides to wild-type levels. We are currently attempting to identify genes or gene products which intervene in the regulation of the holo-PYC production by AniA by isolating mutants of strain VEM58 which have lost the ability to grow on pyruvate.

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