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Mutational Inactivation of a Gene Homologous to *Escherichia coli ptsP* Affects Poly-β-Hydroxybutyrate Accumulation and Nitrogen Fixation in *Azotobacter vinelandii*

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Strain DS988, an *Azotobacter vinelandii* mutant with a reduced capacity to accumulate poly-β-hydroxybutyrate, was isolated after mini-Tn*5* mutagenesis of the UW136 strain. Cloning and nucleotide sequencing of the affected locus revealed a gene homologous to *Escherichia coli ptsP* which encodes enzyme I^{Ntr}, a homologue of enzyme I of the phosphoenol pyruvate-sugar phosphotransferase system with an N-terminal domain similar to the N-terminal domain of some NifA proteins. Strain DS988 was unable to grow diazotrophically with 10 mM glucose as a carbon source. Diazotrophic growth on alternative carbon sources such as gluconate was only slightly affected. Glucose uptake, as well as glucose kinase and glucose-6-phosphate-dehydrogenase activities that lead to the synthesis of gluconate-6-phosphate, were not affected by the *ptsP* mutation. The inability of DS988 to grow diazotrophically in 10 mM glucose was overcome by supplying ammonium or other sources of fixed nitrogen. Acetylene reduction activity but not transcription of the nitrogenase structural gene *nifH* was shown to be impaired in strain DS988 when it was incubated in 10 mM glucose. The diazotrophic growth defect of DS988 was restored either by increasing the glucose concentration to above 20 mM or by lowering the oxygen concentration. These data suggest that a mutation in *ptsP* leads to a failure in poly-β-hydroxybutyrate metabolism and in the respiratory protection of nitrogenase under carbon-limiting conditions.

Azotobacter vinelandii is an obligate, aerobic, nitrogen-fixing soil bacterium that undergoes differentiation by forming desiccation-resistant cysts and produces the intracellular polyester poly-β-hydroxybutyrate (PHB). Oxygen limitation initiates the synthesis of this polymer (31). Under relaxed oxygen conditions, acetyl-coenzyme A (CoA) is fed into the tricarboxylic acid cycle, and the resultant CoA inhibits the β-ketothiolase activity, which catalyzes the first step of PHB synthesis. Under oxygen limitation and carbon excess, NADPH increases and inhibits citrate synthase and isocitrate dehydrogenase, raising the levels of acetyl-CoA and lowering the CoA levels; thus, the inhibition of the β-ketothiolase by CoA is overcome, allowing synthesis of PHB to proceed (32).

A. vinelandii fixes nitrogen under fully aerobic growth conditions due to protection of its oxygen labile nitrogenase from inactivation. Protection of nitrogenase is achieved by two mechanisms. In the first, called respiratory protection, *A. vinelandii* can exhibit one of the highest known respiration rates at the expense of a high rate of carbon and energy source consumption, maintaining a low intracellular oxygen concentration. In the second mechanism, called conformational protection, when oxygen stress occurs nitrogenase undergoes a conformational switch to a reversible inactive but protected state, a process mediated by the FeSII protein (26).

A. vinelandii can grow on a wide variety of carbon sources under diazotrophic conditions (38) and transports carbohydrates by an active transport mechanism. Glucose transport is coupled to the oxidation of L-malate via the respiratory chain (3). D-Glucose is metabolized via the Entner-Doudoroff pathway (4, 17, 34).

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The phosphoenolpyruvate (PEP):sugar phosphotransferase system (PTS) mediates the uptake and concomitant phosphorylation of many carbohydrates in a number of bacterial genera. Several phosphoryl-transfer proteins catalyze the relay of phosphate from PEP to the incoming sugar. Enzyme I and Hpr, encoded by *ptsI* and *ptsH*, respectively, comprise the soluble PTS proteins and transfer phosphate from PEP to all of the sugar-specific phosphoryl-carrier proteins and are called the general or energy coupling PTS proteins. The enzyme II complexes are carbohydrate specific and are composed of three or four domains organized either as individual polypeptides or as fused proteins, at least one of which is localized in the cytoplasmic membrane (19).

The glucose PTS is widely distributed in genera that are obligate or facultative anaerobes, most frequently in those that ferment glucose via the Embden-Meyerhoff-Parnas pathway, but is absent in bacteria that are strictly aerobic, such as *Pseudomonas*, *Alcaligenes*, and *Azotobacter* spp. (27, 28). However, a fructose PTS is present in *Pseudomonas* and *Alcaligenes* spp., where a fructose-1-phosphate kinase activity enables these bacteria to metabolize this sugar by the Embden-Meyerhoff-Parnas pathway (28). A fructose-1 kinase activity has not been detected in *Azotobacter* spp. (1, 33).

The PTS is not only involved in the transport and phosphorylation of carbohydrates, but it also regulates several metabolic processes, such as catabolism of carbon sources (PTS and non-PTS) by the interrelated phenomena of catabolite repression and inducer exclusion (19).

Genes encoding proteins homologous to PTS components that seem to be involved in other aspects of bacterial physiology have been reported in several bacterial species (21–23, 35). Examples of these *pts* genes include *phbH* and *phbI*, which are present in *Alcaligenes eutrophus*, where they control accumulation of the reserve polymer PHB (21). Other examples of *pts* genes are *ptsN* and *npr* (*ptsO*) of *Escherichia coli* and *Klebsiella*

pneumoniae, encoded within the *rpoN* operon, whose products are homologous to enzyme IIA^{Fru} (IIA^{Ntr}) and Hpr (Npr), respectively, and very likely regulate induction of σ^{54} (RpoN)-controlled promoters. In *E. coli*, IIA^{Ntr} acts to regulate assimilation of nitrogen derived from organic sources (20). In *K. pneumoniae*, certain *ptsN* mutations increase the expression of the p*nifH*, p*nifL*, and p2*glnA*, σ^{54}-dependent promoters, whereas *ptsO* mutations decrease the expression of these promoters, suggesting that unphosphorylated IIA^{Ntr} negatively regulates σ^{54} (15). Since RpoN is the alternative σ^{54} involved in the transcription of genes related to nitrogen metabolism (among other physiological functions), it has been postulated that IIA^{Ntr} and Npr could jointly function as a carbon-nitrogen coordinator (23). The enzyme I responsible for the phosphorylation of Npr and IIA^{Ntr} has not been identified. An *E. coli* gene called *ptsP*, which encodes an enzyme I homologue called enzyme I^{Ntr} with an N-terminal domain homologous to the N-terminal domains of some NifA proteins, has been proposed to be the Npr phosphorylating enzyme (25), but no experimental evidence has been provided.

The results reported here show the presence of a *ptsP* gene in *A. vinelandii* and provide evidence suggesting that its product is involved in the regulation of PHB metabolism and in the respiratory protection of nitrogenase under carbon-limiting conditions.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The *A. vinelandii* strains used in this study were UW136 (5) and MV101 (36). *E. coli* DH5α was used for the isolation and maintenance of plasmids. *E. coli* SM10 (λpir)/pUT–mini-Tn*5*–*lacZ* (7), was used as the donor strain for Tn*5* mutagenesis.

A. vinelandii cells were grown in Burk's medium supplemented with 2% sucrose (BS) or other carbon sources as indicated. Liquid cultures were carried out in 125-ml flasks, containing 25 ml of medium, on a rotatory shaker at 250 rpm and 30°C. In all growth experiments, the inoculum was grown 30 h in BS, washed twice with Burk's buffer, and then transferred to the indicated medium. Growth is reported as the optical density at 600 nm. Identification of mutant DS988 was carried out on peptone-yeast (PY)-rich medium supplemented with 2% sucrose. The same medium, containing 2% of the indicated carbon source instead of sucrose, was used for some PHB determinations. The antibiotics and concentrations (in micrograms per milliliter) used were as follows: nalidixic acid, 20; kanamycin, 3; rifamicin, 10; tetracycline, 10; and spectinomycin, 50.

Transposon mutagenesis. Random transposon mutagenesis of UW136 was carried out as described, with a pUT derivative containing the mini-Tn*5 lacZ2* transposon (7).

Bacterial matings. An *A. vinelandii* library constructed on pCP13 (13) was mobilized from *E. coli* DH5α to DS988 mutant in a triparental mating by using the helper plasmid pRK2013 (9) and selection on BS supplemented with kanamycin and tetracycline.

Enzyme assays. Crude extracts for enzyme determination were prepared as follows. Cultures were centrifuged, and the harvested cells were washed twice with the appropriate buffer. The cells were resuspended in 30 mM Tris-HCl buffer (pH 8.2) for glucose kinase and glucose-6-phosphate (P) dehydrogenase determinations, or in 100 mM Tris-HCl (pH 7.88) for β-ketothiolase determinations, with subsequent cell disintegration by ultrasonic treatment at 5°C. Sonicated cell suspensions were centrifuged at 14,000 g for 10 min. For glucose kinase and glucose-6-P dehydrogenase determinations, the supernatants were centrifuged for 1.5 h at 192,000 g.

β-Ketothiolase enzyme was assayed by its thiolysis activity as described by Senior and Dawes (32). The glucose kinase activity was assayed by coupling glucose-6-phosphate dehydrogenase and then measuring the reduction of NADP at 340 nm according to the method of Angell et al. (2). For glucose-6-phosphate dehydrogenase, the reduction of NADP was measured at 340 nm by the method described by Lessie and Vander Wyk (12). Nitrogenase activity was determined in whole cells by the acetylene reduction assay, as described by Bishop et al. (6). β-Galactosidase activity was determined as described by Miller (16).

Glucose uptake. Glucose transport was measured in cells incubated for 3 h in Burk's medium with 11 mM glucose as the carbon source. The cells were washed twice with cold Burk's buffer, resuspended at a concentration of 0.4 mg (dry weight) ml^{-1}, and incubated in a reaction mixture containing 0.5 mM [^{14}C]glucose (1 mCi $mmol^{-1}$) in Burk's buffer. Then 0.5-ml samples were removed at 0, 3, 6, 9, 12, and 15 min; the samples were filtered through Millipore HA filters (0.45-μm pore size) and washed once with Burk's buffer containing 100 mM unlabeled glucose and twice with cold Burk's buffer. The dried filters were counted in a liquid scintillation counter.

Determination of PHB. The PHB content of the bacteria was determined by the spectrophotometric method of Law and Slepecky (11).

DNA manipulations. Standard procedures for restriction endonuclease digestion, agarose gel electrophoresis, purification of DNA from agarose, and DNA ligation were carried out as described by Sambrook et al. (29). DNA sequences were determined by the dideoxy-chain termination method of Sanger et al. (30).

Construction of strain DS989. A 3.3-kb *Bgl*II DNA fragment containing the 5′ region of the *ptsP* gene from *A. vinelandii* UW136 was cloned into plasmid pUC19 to produce pDS20. A 2-kb *Sma*I fragment containing a tetracycline-resistant gene (*Tc*) from plasmid pHP45Ω-Tc (8) was inserted into the unique *Xho*I site present within the *ptsP* gene in pDS20 to create a *ptsP*::*Tc* mutation within the codon for amino acid residue 224 of enzyme I^{Ntr}. The resultant plasmid pDS20A (Fig. 1), which is unable to replicate in *A. vinelandii*, was introduced by transformation into strain MV101, a UW136 derivative carrying a *nifH*::*lacZ* gene fusion (36). One tetracycline-resistant transformant which was less opaque than MV101, strain DS989, was chosen for further analysis. The substitution of the intact *ptsP* gene with the *ptsP*::*Tc* mutation on the chromosome of the DS989 mutant was confirmed by Southern blotting (data not shown).

Construction of plasmids pDS226 and pDS226b. Oligonucleotides 5′-AG CAGAAGTGGTTCTCCTGC-3′ and 5′-GCATGACCCGCTCGAAGTCTT-3′ and plasmid pDS18, containing the *ptsP* gene in a 6.4-kb *Eco*RI-*Cla*I fragment (Fig. 1), were used to clone the *ptsP* gene by PCR. The resultant 2.8-kb fragment was cloned into the unique *Sma*I site of plasmid pUCP20 (37), producing plasmid pDS226 (Fig. 1). An *Eco*RI-*Hin*dIII fragment containing the *ptsP* gene from pDS226 was cloned into pBR329. The resultant plasmid was used to introduce an Ω-spectinomycin cassette (8) into the *Eco*RI site to produce plasmid pDS226b, which is shown in Fig. 1.

Nucleotide sequence accession number. The nucleotide sequence of the *ptsP* gene reported here has been deposited in the EMBL GenBank and DDBJ Nucleotide Sequence Databases under accession number Y14681.

RESULTS

Isolation of strain DS988. In an attempt to isolate mutants affected in the production of PHB in *A. vinelandii*, we carried out random mini-Tn*5* mutagenesis of strain UW136. This mutagenesis produced strain DS988, which is less opaque than UW136 when grown for 4 days in PY medium plates supplemented with 2% sucrose (Fig. 2). This phenotype is due to a decreased PHB accumulation, since under this condition strain UW136 produced 936 ± 23 μg of PHB per mg of protein, whereas strain DS988 produced 83 ± 46 μg. A kinetic analysis of PHB accumulation during growth of strains UW136 and DS988 in liquid PY-sucrose is shown in Fig. 3. PHB accumulation started in the prestationary phase in both UW136 and DS988; in DS988 it stopped at 32 h, whereas in UW136 it continued during the stationary phase. In UW136 the level of PHB accumulation was 1.8-fold higher in liquid than in solid PY-sucrose cultures. The differences in PHB accumulation between UW136 and DS988 were 11-fold in solid cultures and 3.5-fold in liquid cultures. The ketothiolase activity, which catalyzes the first enzymatic step in PHB biosynthesis, was determined in UW136 and DS988 incubated during 48 h in PY-sucrose liquid cultures; this activity was about 40% of that present in the wild-type strain UW136 (Table 1).

Cloning and DNA sequence. An *A. vinelandii* cosmid gene library was introduced by conjugation into strain DS988. Two cosmid clones pMS620 and pMS3405 that restored the opacity in strain DS988 were identified.

An 8.2-kb *Pst*I fragment containing the 5.0-kb mini-Tn*5* from DS988 mutant was cloned into pBluescript. The resultant plasmid, pDS2t, hybridized to a 3.2-kb *Pst*I fragment of UW136 DNA and to cosmids pMS620 and pMS3405 harboring the wild-type opacity-complementing region.

The 3.2-kb *Pts*I and 6.4-kb *Eco*RI-*Cla*I restriction fragments from plasmid pMS620 that hybridized with plasmid pDS2t were cloned. A restriction map of the resultant plasmids pDS2 and pDS18 is shown in Fig. 1. The DNA sequence of a 3-kb region in these plasmids revealed the presence of one open reading frame (ORF) encoding a polypeptide of 759 amino acid residues (Fig. 4), with a calculated molecular weight of

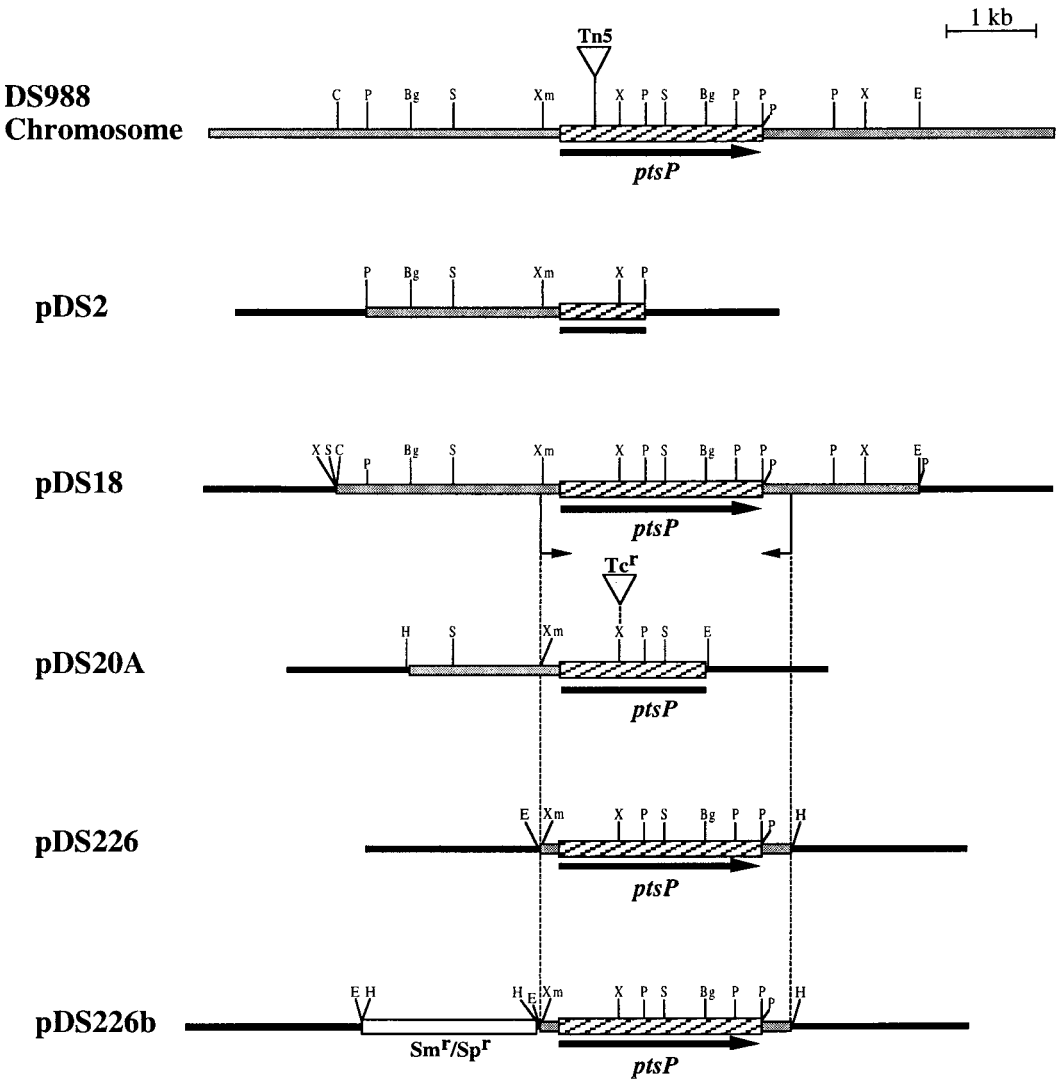

FIG. 1. Physical map of the *A. vinelandii ptsP* region and plasmids constructed in this work. The *ptsP* gene is represented by the arrow. The transposon insertion site is indicated. Vector sequences are represented by black bars. Restriction site abbreviations: Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; X, *Xho*I; Xm, *Xmn*I.

83,640. The exact location of the Tn*5* mutation was determined by nucleotide sequencing across the transposon insertion junction and was found to lie within codons 130 and 131 of this ORF (Fig. 4). A database search with the amino acid sequence of this ORF established a high degree of similarity with enzyme I proteins of the PEP PTS and, specifically, an overall 43% identity with enzyme I^{Ntr} encoded by the *ptsP* gene of *E. coli* (25). Accordingly, this ORF was designated *ptsP*. The *A. vinelandii ptsP* gene product has an N-terminal domain of 160 amino acids, showing a high degree of identity with the N-terminal domain of the NifA nitrogen fixation regulators of *Azospirillum lipoferum*, *Azospirillum brasiliense*, and *Herbaspirillum seropedicae*, as well as AnfA and VnfA of *A. vinelandii* (Swiss-Prot accession numbers P54929, P30667, P27713, P12626, and P12627, respectively). As in the *E. coli* enzyme I^{Ntr}, in the *A. vinelandii* protein a putative Q linker is present at the boundaries of the N-terminal and C-terminal domains (Fig. 4, amino acids 158 to 177). The phosphorylation site signature of PEP-utilizing enzymes G-[GA]-x-[TN]-x-H-[STA]-[STAV]-[LIVM](2)-[STAV]-R (Prosite name PS00370) is present at positions 358 to 369 (GSGNSHVAILAR), with the histidyl residue involved in the phosphorylation at position 363. This signature does not correspond exactly to the reported signature for this motif in positions 359 ([GA] > S) and 364 ([STA] > V). We propose the following modification for the signature of the phosphorylation site in PEP-utilizing enzymes: G-[GAS]-

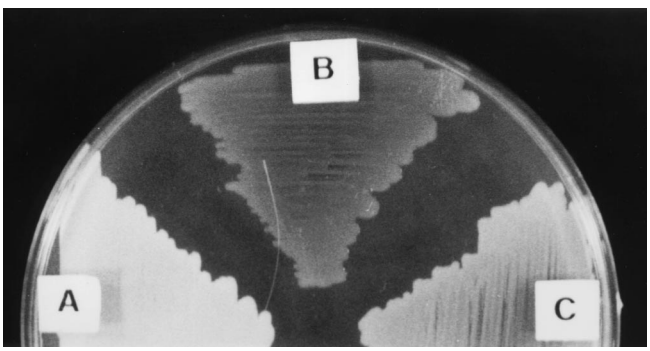

FIG. 2. Opacity phenotypes of *A. vinelandii* UW136 (A), DS988 (B), and DS988::pDS226b (C) grown on PY sucrose plates during 5 days.

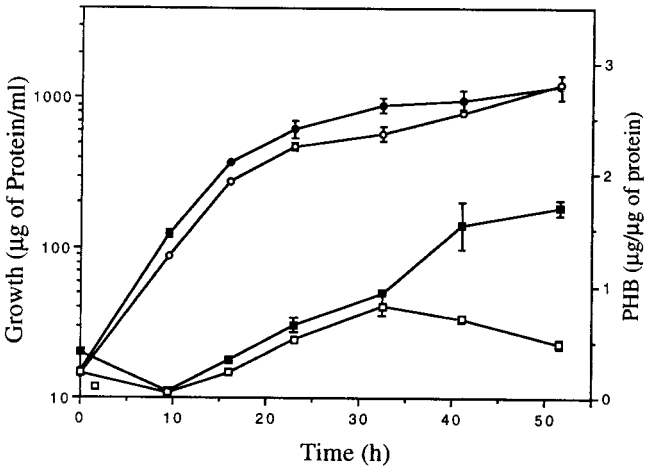

FIG. 3. Growth (circles) and PHB accumulation (squares) kinetics by UW136 (solid symbols) and DS988 (open symbols) strains.

x-[TN]-x-H-[STAV](2)-[LIVM](2)-[STAV]-R, since only proteins belonging to this family were identified in release 34 of the SwissProt data bank with this modified signature.

A second signature, one typical of proteins of the family of PEP-utilizing enzymes, [DES]-x-[LIVMF]-2-[LIVMF]-G-[ST]-N-D-[LIVM]-x-Q-[LIVMFYG]-[STALIV]-[LIVMF]-[GAS]-x(2)-R (Prosite name PS00742), is present within amino acids 620 to 638 (DFLSVGSNDLTQYLLAVDR) of *A. vinelandii* enzyme I^{Ntr}. A cysteinyl residue, proposed to be implicated in the active site and conserved in all members of this family (24), is present at position 675.

Growth and PHB accumulation on different carbon sources. The *pts* genes are involved in the transport and phosphorylation of sugars, as well as in the regulation of the assimilation of carbon sources (19). Although *A. vinelandii* does not transport glucose by this system, we tested the ability of strain DS988 to grow diazotrophically in nitrogen-free Burk's medium with different carbon sources, including sugars and intermediates of the tricarboxylic acid cycle. Strain DS988 grew in all of the carbon sources tested, except on 10 mM glucose or 50 mM glycerol (Fig. 5). A longer lag phase was observed in DS988 grown on sucrose, gluconate, or mannitol.

We also determined the effect of the carbon source on the accumulation of PHB by DS988. Strain DS988 was grown diazotrophically on solid Burk's medium supplemented with 2% fructose, gluconate, glucose, or pyruvate as the sole carbon source. As seen in Fig. 6, PHB accumulation substantially diminished in all of the carbon sources tested. Similar results were obtained when the strain was grown in solid PY-rich medium supplemented with the above-mentioned carbon sources (data not shown).

Uptake, phosphorylation, and catabolism of glucose is not affected by the *ptsP* mutation. In *A. vinelandii* the Entner-Doudoroff pathway is the major route for glucose catabolism (4). This, along with the fact that growth on gluconate was not impaired, lead us to hypothesize that either glucose uptake or the first two steps in the Entner-Doudoroff pathway leading to the formation of gluconate-6-phosphate could be affected by the *ptsP* mutation. Table 1 shows that this is not the case, since both glucose uptake and the glucose kinase and glucose-6-phosphate dehydrogenase activities in DS988 are similar to those of the wild type.

The *ptsP* mutation affects nitrogen fixation. Since glucose catabolism seems not to be affected in strain DS988 and since growth inhibition on glucose is observed in nitrogen-free Burk's medium, the possibility that the *ptsP*::Tn*5* mutation affected nitrogen fixation was tested. Nitrogenase activity, determined by acetylene reduction assays, was not detected in strain DS988 when incubated in Burk's medium supplemented with 10 mM glucose and was reduced by about 50% in the same medium supplemented with 10 mM gluconate (Table 1). In the wild-type strain UW136 this activity was found to be 9 times lower in Burk's medium supplemented with 10 mM glucose than in the same medium supplemented with 10 mM gluconate (Table 1). Furthermore, growth on 10 mM glucose was restored by the addition of 10 mM fixed nitrogen source such as ammonium chloride, alanine, asparagine, glutamate, glutamine, or urea (data not shown).

To determine whether the effect of the *ptsP* mutation on nitrogen fixation was at the level of transcription of the *nif* structural genes, we constructed, as described in Materials and Methods, strain DS989 (an MV101 derivative, with a *ptsP*::*Tc* mutation, which is in turn a UW136 derivative carrying a *nifH*::*lacZ* gene fusion). β-Galactosidase activities, determined after 4 h of incubation on Burk's glucose (inducing condition) and Burk's glucose supplemented with ammonium chloride (non-inducing condition), were similar in strains MV101 and DS989 (Table 2). Thus, no effect on transcription of the *nifH* gene by the *ptsP* mutation, as measured by galactosidase activity, was observed.

Glucose concentrations above 20 mM or oxygen limitation restore diazotrophic growth. In *A. vinelandii* cultures, carbon limitation increased the oxygen sensitivity of nitrogenase (10). Since the growth-deficient phenotype of the DS988 mutant was observed on the carbon sources that gave the lowest growth rates (glucose and glycerol), we hypothesized that the effect on nitrogenase activity could be attributed to a defective nitrogenase protection. Diazotrophic growth of strain DS988 was restored when the glucose concentration in the medium was increased to more than 20 mM (Fig. 7A). In a similar way, the lag on gluconate was overcome by increasing its concentration in the medium (data not shown). Growth of DS988 on 10 mM glucose was also restored when the aeration of the culture was

TABLE 1. Effect of *ptsP* mutation on different enzymatic activities[a]

Strain	Activity					
	Glucose uptake (nmol min^{-1} mg^{-1})	Glucose kinase (nmol min^{-1} mg^{-1})	Glucose-6-phosphate dehydrogenase (nmol min^{-1} mg^{-1})	Nitrogenase on glucose (nmol h^{-1} g^{-1})	Nitrogenase on gluconate (nmol h^{-1} g^{-1})	β-Ketothiolase (nmol h^{-1} g^{-1})[b]
UW136	4.01 ± 0.56	19.7 ± 2	311.2 ± 10.7	1,096 ± 50	9,097 ± 260	2,980 ± 138
DS988	5.02 ± 0.31	18.8 ± 3.2	256.1 ± 48.0	ND[c]	4,200 ± 612	1,223 ± 36

[a] Cells were grown for 20 h on BS, washed with the same medium without a carbon source, and incubated for 4 h on Burk's medium supplemented with 11 mM glucose or gluconate. Values are the means of three determinations.
[b] β-Ketothiolase activity was determined from cells grown for 48 h in liquid PY-sucrose medium.
[c] ND, not detected.

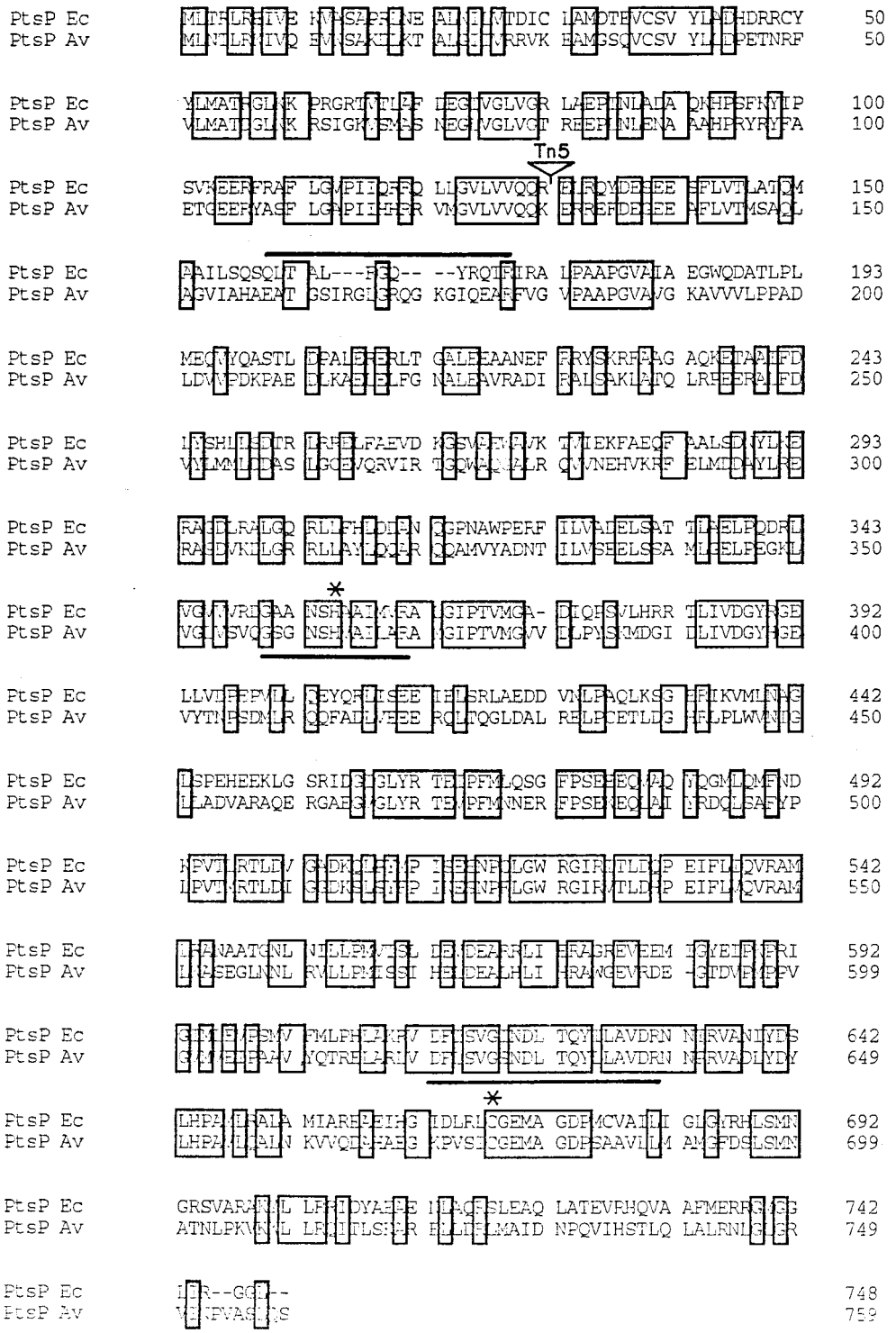

FIG. 4. Alignment of the deduced amino acid sequences of PtsP from *E. coli* (Ec) and *A. vinelandii* (Av). Identical residues are boxed. Histidine involved in phosphorylation and the active site cysteine are marked by asterisks. The signatures of PEP-utilizing enzymes are underlined. The Q linker is overlined. The arrow denotes the position of the Tn*5* insertion.

lowered by increasing the volume of medium in the flasks (Fig. 7B). These results are consistent with the hypothesis of a failure in the respiratory protection of nitrogenase in DS988.

The PHB and nitrogen fixation-deficient phenotypes are caused by the *ptsP*::Tn*5* mutation. Cosmids pMS620 and pMS3405 restored the wild-type colony opacity and the ability to grow on glucose with N_2 (data not shown), suggesting that the phenotypes described are caused by the *ptsP*::Tn*5* mutation. Plasmid pDS226 (Fig. 1), which is able to replicate in *A. vinelandii* and carries the *ptsP* gene flanked by 215 bp up-

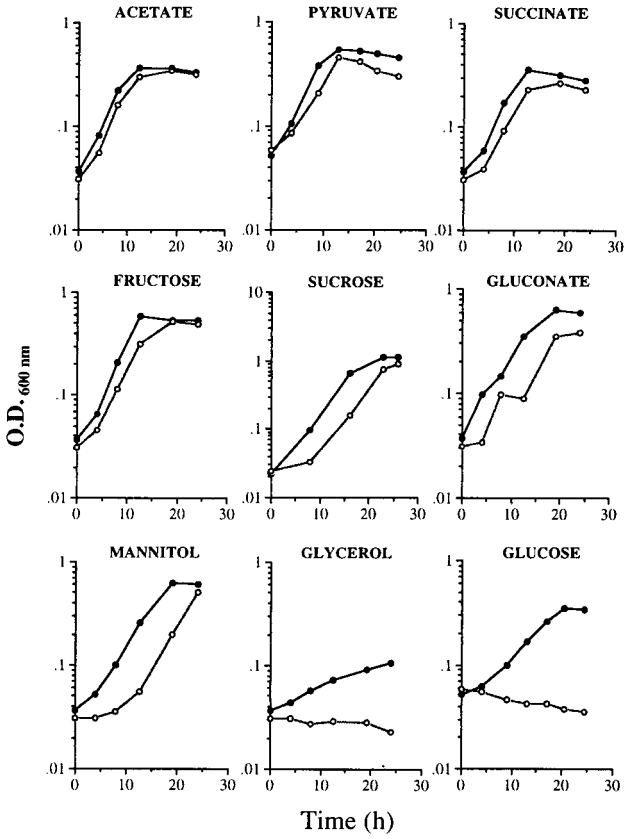

FIG. 5. Growth of *A. vinelandii* strains UW136 (●) and DS988 (○) on Burk's medium supplemented with different carbon sources. The cultures were pregrown on BS. The concentration for all carbon sources tested was 10 mM, except for acetate (20 mM) and glycerol (50 mM). The data are representative of three different experiments.

stream of the ATG start codon and 322 bp downstream of the stop codon, failed to complement the opacity and nitrogen fixation phenotypes of strain DS988, suggesting that the promoter transcribing *ptsP* is not present in this plasmid or that the phenotype in DS988 is due to a polar effect on genes downstream of *ptsP*. The fragments containing the *ptsP* gene, as well as a spectinomycin gene, were cloned into plasmid pBR329; the resultant plasmid pDS226b (Fig. 1), which is unable to replicate in *A. vinelandii*, was transformed into DS988 for integration into the chromosome. Two types of ampicillin- and spectinomycin-resistant transformants were selected: those that showed the wild-type colony opacity (DS988::pDS226b, Fig. 2) and the ability to grow on N_2 in Burk's medium with 10 mM glucose and those with the DS988 opacity phenotype that were unable to grow on N_2 in Burk's medium with 10 mM glucose. Southern blot analysis (Fig. 8) showed that integration of pDS226b in a transformant (DS988::pDS226b) with the wild-type phenotype occurred between the *ptsP* promoter region and the Tn*5* insertion, thus allowing the wild-type *ptsP* gene to be transcribed from its own promoter (Fig. 8B). These data confirm that the PHB and nitrogen fixation phenotypes are due to the *ptsP* mutation and not to a polar effect.

DISCUSSION

Characterization of a mutant affected in its ability to accumulate PHB allowed us to establish the presence in *A. vinelandii* of a *ptsP* gene encoding an enzyme I^{Ntr} homologue that

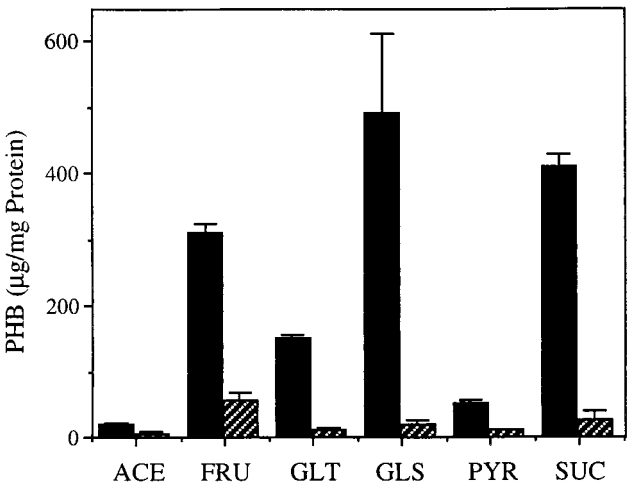

FIG. 6. PHB accumulation by *A. vinelandii* UW136 (solid bars) and DS988 (striped bars) grown on solid Burk's medium supplemented with 2% different carbon sources: ACE, acetate; FRU, fructose; GLT, gluconate; GLS, glucose; PYR, pyruvate; and SUC, sucrose.

has been recently described in *E. coli*. Since *A. vinelandii* has been reported to lack an active PEP:glucose phosphotransferase (28) and to transport glucose by another mechanism (3), the possibility that this enzyme I^{Ntr} could participate in the transport of other carbohydrates such as fructose, as is the case in *Pseudomonas* spp. (28), was raised. However, the *ptsP* mutation did not markedly affect fructose utilization (Fig. 5), a finding in agreement with the absence in *A. vinelandii* of fructose-1-phosphate kinase activity (1); this is usually associated with PTS-dependent fructose transport in aerobic bacteria (28). Because the assimilation of other carbohydrates was also unaffected, it is unlikely that the *A. vinelandii* enzyme I^{Ntr} participates in the transport of carbohydrates or in the regulation of its catabolism.

It has been suggested that in *E. coli*, enzyme I^{Ntr} participates in a phosphate relay with Npr and IIA^{Ntr} proteins (25). In fact, Npr can be a phosphate acceptor from PTS enzyme I-P, although this phosphorylation is less efficient than that of Hpr, and Npr-P is able to transfer the phosphate to IIA^{Ntr} in vitro (20). It has also been demonstrated that IIA^{Ntr} of *K. pneumoniae* can be a substrate of Hpr-P and that an *npr* mutation diminishes transcription of σ^{54}-dependent promoters in this bacterium, implying that unphosphorylated IIA^{Ntr} negatively regulates σ^{54}. Enzyme I^{Ntr} is present in *A. vinelandii* (this study), and although the presence of *npr* and *ptsN* homologues in *A. vinelandii* has not been demonstrated, sequence analysis of the *rpoN* region suggested the presence of these genes in this bacterium (14). We show here that although a *ptsP* mutation

TABLE 2. Effect of *ptsP* mutation on transcription of *nifH*[a]

Strain	Genotype	β-Galactosidase sp act (Miller units) at:		
		0 h	4 h	
			Without ammonium	With ammonium
MV101	*ptsP* (wild type)	135.5 ± 11.2	1,392.9 ± 125	82.4 ± 1
DS989	*ptsP*	154.4 ± 51.8	1,219.0 ± 111	79.2 ± 7

[a] Cells were grown for 20 h on BS and 10 mM urea, washed with Burk's buffer, and incubated for 4 h on Burk's medium supplemented with 11 mM glucose with or without 10 mM ammonium chloride. Values are the means of three determinations.

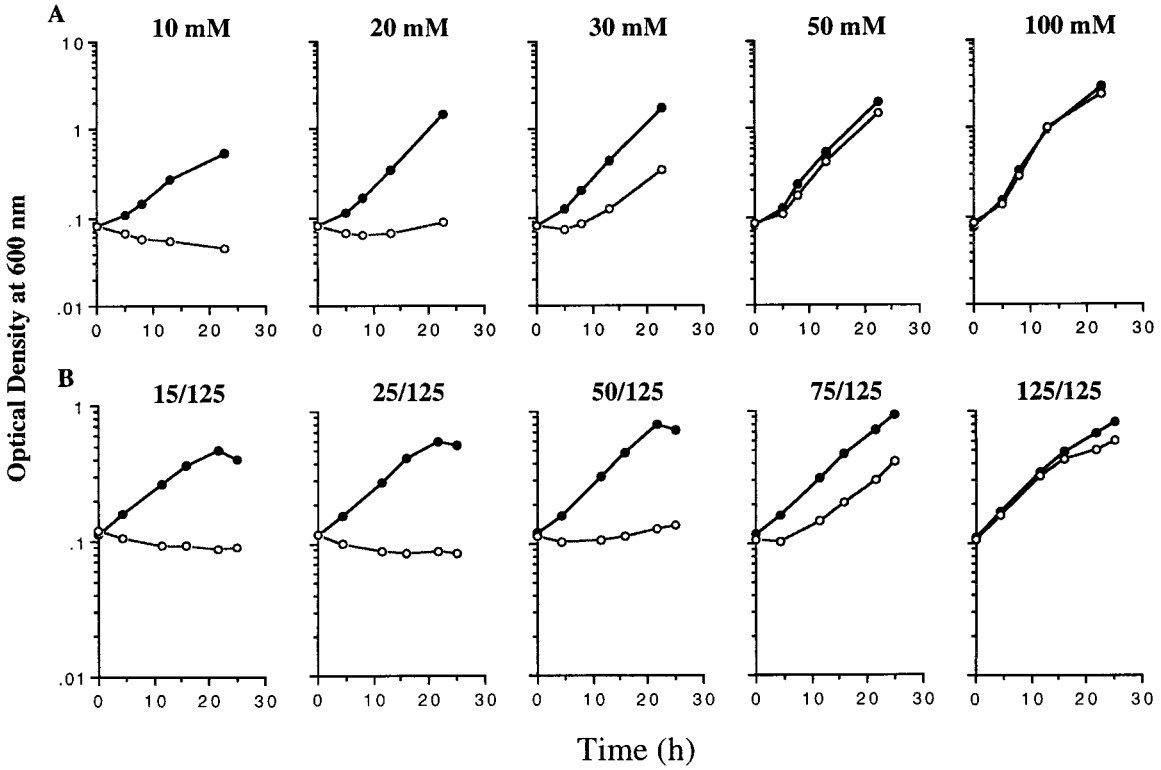

FIG. 7. Effect of glucose (A) or oxygen (B) concentration on the growth of *A. vinelandii* strains UW136 (●) and DS988 (○). Numbers in panel B indicate the volumes of medium used in 125-ml flasks. The data are representative of two different experiments.

negatively affects nitrogenase activity, it does not affect transcription from the *nifH* σ^{54}-dependent promoter, implying that in *A. vinelandii*, the enzyme I^{Ntr} does not participate in the control of this σ^{54}-dependent promoter.

The point at which nitrogenase activity is affected in strain DS988 seems to be a failure in the respiratory protection under carbon-limiting conditions, where oxygen-consuming respiratory protection is restricted. We provided evidence supporting

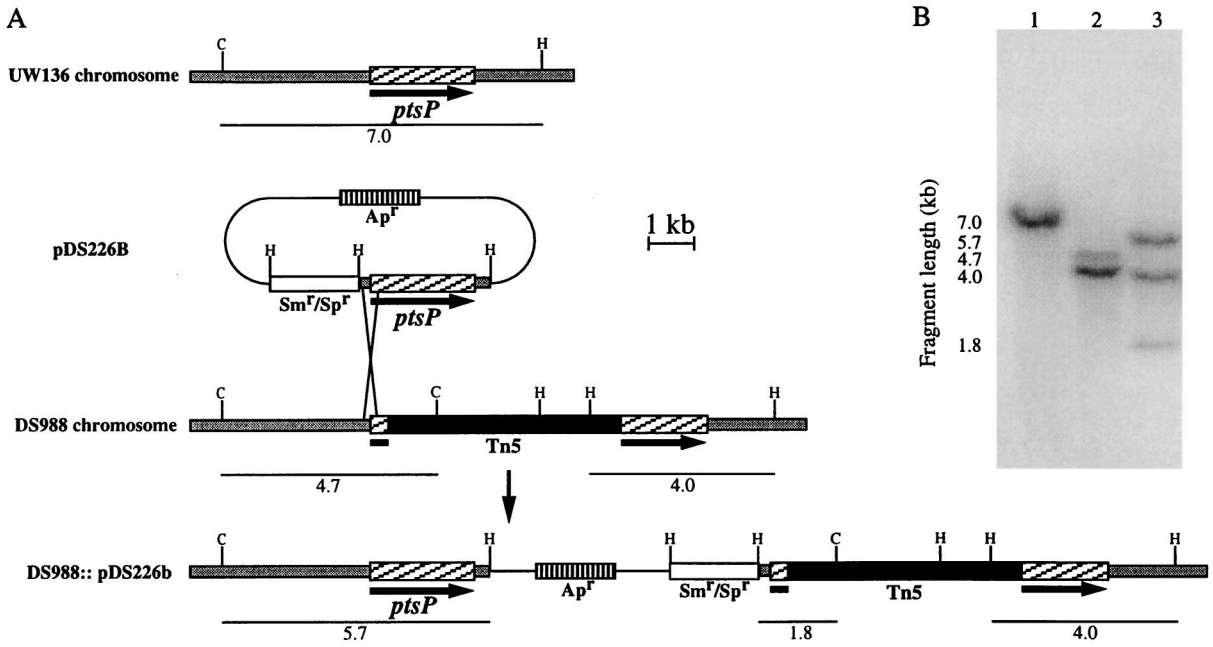

FIG. 8. Integration of plasmid pDS226b into the chromosome of strain DS988. (A) Schematic representation of the pDS226b integration into the DS988 chromosome. (B) Southern blot hybridization of total genomic DNA from UW136 (lane 1), DS988 (lane 2), and DS988::pDS226b (lane 3) digested with *Cla*I and *Hin*dIII with *ptsP* as probe. The hybridizing fragments in panel B are as indicated in panel A.

this proposal, since increasing the glucose concentration above 10 mM reestablished the diazotrophic growth of the mutant. It has been proposed that the nitrogenase complex is more sensitive to oxygen inactivation upon energy (carbon) starvation due to a reduced flux of electrons to the complex (10). By lowering the oxygen concentration, the nitrogenase inhibition in DS988 was also overcome.

Upon energy starvation, the conformational protection mediated by the FeSII protein temporarily protects nitrogenase from inactivation and subsequent degradation (18); therefore, it is also possible that the conformational protection by FeSII is affected in DS988. It would be interesting to test a *fesII* mutant for growth on BS 10 mM glucose. These interpretations imply that glucose is a poor carbon source for *A. vinelandii*. The doubling time of strain UW136 on glucose under diazotrophic conditions is significantly longer than that observed when grown on gluconate or fructose at the same molar concentrations (Fig. 5). In addition, the high-energy-demanding nitrogenase activity was found to be nine times lower on glucose than that on gluconate (Table 1), and it has been shown that there is a relationship between nitrogenase activity and the supply of carbon source (10). These data are consistent with glucose being an energy-limiting carbon source.

The effect of the *ptsP* mutation on nitrogenase activity could also be a consequence of the reduced capacity for PHB accumulation. It has been suggested that PHB participates in the regulation of the intracellular oxygen environment by providing a readily oxidized carbon source that could increase the oxidative activity in the absence of exogenous substrate, thus facilitating the respiratory protection of nitrogenase (31).

The control point at which PHB accumulation is affected in DS988 is not known; involvement of PTS homologous proteins in the regulation of PHB metabolism has been reported in *A. eutrophus*, where mutations in either *ptsI* (*phbI*) or *ptsH* (*phbH*) genes (encoding enzyme I and Hpr homologues) caused the polymer content to decrease more rapidly than in the wild type. In addition, the opacity of the mutants decreased further after prolonged incubation (21). Thus, the decrease in PHB content seems to be due to PHB degradation. Our data do not rule out the possibility that, in *A. vinelandii*, the decrease in PHB content caused by the *ptsP* mutation is due to degradation. However, we observed neither a decrease in opacity after prolonged incubation nor a drastic decrease in PHB. Furthermore, the lower β-ketothiolase activity observed in the DS988 cells (Table 1) implies that it may be due to a decrease in its synthesis. The reduction of PHB is higher than the reduction in the ketothiolase activity; this could be explained by the presence of two ketothiolase activities in *A. vinelandii* (30a).

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REFERENCES

1. **Anderson, A. J., A. J. Hacking, and E. A. Dawes.** 1987. Alternative pathways for the biosynthesis of alginate from fructose and glucose in *Pseudomonas mendocina* and *Azotobacter vinelandii*. J. Gen. Microbiol. **133:**1045–1052.
2. **Angell, S., E. Schwarz, and M. J. Bibb.** 1992. The glucose kinase gene of *Streptomyces coelicolor* A3 (2): its nucleotide sequence, transcriptional analysis and role in glucose repression. Mol. Microbiol. **6:**2833–2844.
3. **Barnes, E. M.** 1972. Respiration-coupled glucose transport in membrane vesicles from *Azotobacter vinelandii*. Arch. Biochem. Biophys. **152:**795–799.
4. **Beale, J. M., and J. L. Foster.** 1996. Carbohydrate fluxes into alginate biosynthesis in *Azotobacter vinelandii* NCIB 8789: NMR investigations of the triose pools. Biochemistry **35:**4492–4501.
5. **Bishop, P. E., and W. J. Brill.** 1977. Genetic analysis of *Azotobacter vinelandii* mutant strains unable to fix nitrogen. J. Bacteriol. **130:**954–956.
6. **Bishop, P. E., D. M. Jarlenski, and D. R. Hetherington.** 1980. Evidence for an alternative nitrogen fixation system in *Azotobacter vinelandii*. Proc. Natl. Acad. Sci. USA **77:**7342–7246.
7. **De Lorenzo, V., M. Herrero, V. Jakubzik, and K. N. Timmis.** 1990. Mini-Tn*5* transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. J. Bacteriol. **172:**6568–6572.
8. **Fellay, R., J. Frey, and H. Krisch.** 1987. Interposon mutagenesis of soil and water bacteria: a family of DNA fragments designed for *in vitro* insertional mutagenesis of Gram-negative bacteria. Gene **52:**147–154.
9. **Figurski, D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. Proc. Natl. Acad. Sci. USA **76:**1648–1652.
10. **Kuhla, J., and J. Oelze.** 1988. Dependence of nitrogenase switch-off upon oxygen stress on the nitrogenase activity in *Azotobacter vinelandii*. J. Bacteriol. **170:**5325–5329.
11. **Law, J. H., and R. A. Slepecky.** 1961. Assay of poly-β-hydroxybutyric acid. J. Bacteriol. **82:**33–36.
12. **Lessie, T. G., and J. C. Vander Wyk.** 1972. Multiple forms of *Pseudomonas multivorans* glucose-6-phosphate and 6-phosphogluconate dehydrogenases. J. Bacteriol. **110:**1107–1117.
13. **Martínez-Salazar, J. M., S. Moreno, R. Nájera, C. Boucher, G. Espín, G. Soberón-Chávez, and V. Deretic.** 1996. Characterization of the genes coding for the putative sigma factor AlgU and its regulators MucA, MucB, MucC, and MucD in *Azotobacter vinelandii* and evaluation of their role in alginate biosynthesis. J. Bacteriol. **178:**1800–1808.
14. **Merrick, M. J., J. Gibbins, and A. Toukdarian.** 1987. The nucleotide sequence of the sigma factor gene *ntrA* (*rpoN*) of *Azotobacter vinelandii*: analysis of conserved sequences in NtrA proteins. Mol. Gen. Genet. **210:**323–330.
15. **Merrick, M. J., M. Taylor, M. H. Saier, and J. Reizer.** 1995. The role of genes downstream of the n N structural gene *rpoN* in *Klebsiella pneumoniae*, p. 189–194. *In* I. A. Tikhonovich, N. A. Provorov, V. I. Romanov, and W. E. Newton (ed.), Nitrogen fixation: fundamentals and application. Kluwer Academic Publishers, Amsterdam, The Netherlands.
16. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 431–435. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
17. **Morteson, L. E., and W. Wilson.** 1955. Initial stages in the breakdown of carbohydrates by *Azotobacter vinelandii*. Arch. Biochem. Biophys. **53:**425–435.
18. **Moshiri, F., J. W. Kim, C. Fu, and R. J. Maier.** 1994. The FeSII protein of *Azotobacter vinelandii* is not essential for aerobic nitrogen fixation, but confers significant protection to oxygen-mediated inactivation of nitrogenase *in vitro* and *in vivo*. Mol. Microbiol. **14:**101–114.
19. **Postma, P. W., J. W. Lengeler, and G. R. Jacobson.** 1993. Phosphoenolpyruvate:carbohydrate phosphotransferase system in bacteria. Microbiol. Rev. **57:**543–594.
20. **Powell, B. S., D. L. Court, T. Inada, Y. Nakamura, V. Michotey, X. Cui, A. Reizer, M. H. Saier, and J. Reizer.** 1995. Novel proteins of the phosphotransferase system encoded within the *rpoN* operon of *Escherichia coli*. J. Biol. Chem. **279:**4822–4839.
21. **Pries, A., H. Priefert, N. Krüger, and A. Steinbüchel.** 1991. Identification and characterization of two *Alcaligenes eutrophus* gene loci relevant to the poly(β-hydroxybutyric acid)-leaky phenotype which exhibit homology to the *ptsH* and *ptsI* of *Escherichia coli*. J. Bacteriol. **173:**5843–5853.
22. **Reizer, J., A. Peterkofsky, and A. H. Romano.** 1988. Evidence for the presence of heat-stable protein (Hpr) and ATP-dependent Hpr kinase in heterofermentative lactobacilli lacking phosphoenolpyruvate:glucose phosphotransferase activity. Proc. Natl. Acad. Sci. USA **85:**2041–2045.
23. **Reizer, J., A. Reizer, M. H. Saier, and G. R. Jacobson.** 1992. A proposed link between nitrogen and carbon metabolism involving protein phosphorylation in bacteria. Protein Sci. **1:**722–726.
24. **Reizer, J., C. Hoischen, A. Reizer, T. N. Pham, and M. H. Saier.** 1993. Sequence analyses and evolutionary relationships among the energy-coupling proteins enzyme I and Hpr of the bacterial phosphoenolpyruvate:sugar phosphotransferase system. Protein Sci. **2:**506–521.
25. **Reizer, J., A. Reizer, M. J. Merrick, G. Plunkett III, D. J. Rose, and M. H. Saier.** 1996. Novel phosphotransferase-encoding genes revealed by analysis of the *Escherichia coli* genome: a chimeric gene encoding an enzyme I homologue that possesses a putative sensory transduction domain. Gene **181:**103–108.
26. **Robson, R. L., and J. R. Postgate.** 1980. Oxygen and hydrogen in biological nitrogen fixation. Annu. Rev. Microbiol. **34:**183–207.
27. **Romano, A. H., S. J. Eberhard, S. L. Dingle, and T. D. McDowell.** 1970. Distribution of the phosphoenolpyruvate:glucose phosphotransferase system in bacteria. J. Bacteriol. **104:**808–813.
28. **Romano, A. H., and M. H. Saier.** 1992. Evolution of the bacterial phosphoenolpyruvate:sugar phosphotransferase system. Section I. Physiological and organismic considerations, p. 143–170. *In* R. P. Mortlock (ed.), Evolu-

tion of metabolic function. CRC Press, Inc., Boca Raton, Fla.
29. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
30a. **Segura, D., E. Vargas, and G. Espín.** Unpublished results.
31. **Senior, P. J., G. A. Beech, G. A. Ritchie, E. A. Dawes.** 1972. The role of oxygen limitation in the formation of poly-β-hydroxybutyrate during batch and continuous culture of *Azotobacter beijerinckii*. Biochem. J. **128:**1193–1201.
32. **Senior, P. J., and E. A. Dawes.** 1973. The regulation of poly-β-hydroxybutyrate metabolism in *Azotobacter beijerinckii*. Biochem. J. **134:**225–238.
33. **Stephenson, M. P., F. A. Jackson, and E. A. Dawes.** 1978. Further observations on carbohydrate metabolism and its regulation in *Azotobacter beijerinckii*. J. Gen. Microbiol. **109:**89–96.
34. **Still, G. G., and C. H. Wang.** 1964. Glucose catabolism in *Azotobacter vinelandii*. Arch. Biochem. Biophys. **105:**126–132.
35. **Titgemeyer, F., J. Walkenhorst, X. Cui, J. Reizer, and M. H. Saier.** 1994. Proteins of the phosphoenolpyruvate:sugar phosphotransferase system in *Streptomyces*: possible involvement in the regulation of antibiotic production. Res. Microbiol. **145:**89–92.
36. **Walmsley, J., and C. Kennedy.** 1991. Temperature-dependent regulation by molybdenum and vanadium of expression of the structural genes encoding three nitrogenases in *Azotobacter vinelandii*. Appl. Environ. Microbiol. **57:** 622–624.
37. **West, S. E. H., H. P. Schweizer, C. Dall, A. K. Sample, and L. J. Runyen-Janecky.** 1994. Contribution of improved *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19 and sequence at the region required for their replication in *Pseudomonas aeruginosa*. Gene **128:**81–86.
38. **Wong, T. Y., and R. J. Maier.** 1985. H_2-dependent mixotrophic growth of N_2-fixing *Azotobacter vinelandii*. J. Bacteriol. **163:**528–533.