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The *cbb* Operons of the Facultative Chemoautotroph *Alcaligenes eutrophus* Encode Phosphoglycolate Phosphatase

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The two highly homologous *cbb* operons of *Alcaligenes eutrophus* H16 that are located on the chromosome and on megaplasmid pHG1 contain genes encoding several enzymes of the Calvin carbon reduction cycle. Sequence analysis of a region from the promoter-distal part revealed two open reading frames, designated *cbbT* and *cbbZ*, at equivalent positions within the operons. Comparisons with known sequences suggested *cbbT* to encode transketolase (TK; EC 2.2.1.1) as an additional enzyme of the cycle. No significant overall sequence similarities were observed for *cbbZ*. Although both regions exhibited very high nucleotide identities, 93% (*cbbZ*) and 96% (*cbbT*), only the chromosomally encoded genes were heterologously expressed to high levels in *Escherichia coli*. The molecular masses of the observed gene products, CbbT (74 kDa) and CbbZ (24 kDa), correlated well with the values calculated on the basis of the sequence information. TK activities were strongly elevated in *E. coli* clones expressing *cbbT*, confirming the identity of the gene. Strains of *E. coli* harboring the chromosomal *cbbZ* gene showed high levels of activity of 2-phosphoglycolate phosphatase (PGP; EC 3.1.3.18), a key enzyme of glycolate metabolism in autotrophic organisms that is not present in wild-type *E. coli*. Derepression of the *cbb* operons during autotrophic growth resulted in considerably increased levels of TK activity and the appearance of PGP activity in *A. eutrophus*, although the pHG1-encoded *cbbZ* gene was apparently not expressed. To our knowledge, this study represents the first cloning and sequencing of a PGP gene from any organism.

The Calvin carbon reduction cycle operates in CO_2 assimilation during autotrophic growth of the aerobic facultative chemoautotroph *Alcaligenes eutrophus*, which oxidizes hydrogen or formate to gain energy and reducing power (6). Several enzymes of the cycle are encoded within the two highly homologous *cbb* operons (formerly *cfx* operons [44]) located on the chromosome and on megaplasmid pHG1 of *A. eutrophus* H16 (7). Both operons form a regulon controlled by the product of regulatory gene *cbbR*, which is divergently oriented to and separated by only 167 bp from the chromosomal *cbb* operon (50). The *cbbLS* genes, encoding the carboxylating enzyme of the Calvin cycle, ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO; EC 4.1.1.39), form the 5′ termini of the *cbb* operons. Among the genes with unknown functions are *cbbXY*, located in the promoter-proximal parts of the operons (27), and *cbbZ*, located in the promoter-distal parts of the operons. The present study focused on the latter gene and its 5′-preceding gene, *cbbT*, which had already been tentatively identified as coding for the Calvin cycle enzyme transketolase (TK; EC 2.2.1.1) (7). On the basis of nucleotide sequencing of the genes and their heterologous expression in *Escherichia coli*, we provide evidence that *cbbT* is in fact a TK gene and that *cbbZ* encodes a key enzyme of the glycolate oxidation pathway in aerobic autotrophs, 2-phosphoglycolate phosphatase (PGP; EC 3.1.3.18). Because of the function of RubisCO as an oxygenase in the presence of molecular oxygen, resulting in the unfavorable formation of 2-phosphoglycolate (31), the organism may take physiological advantage of the inclusion of the PGP genes within the *cbb* operons. There has been no previous report on the molecular analysis and identification of a PGP gene from any organism.

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MATERIALS AND METHODS

Strains, plasmids, and culture conditions. Table 1 lists the bacterial strains and plasmids used in this study. Strains of *A. eutrophus* were grown as described previously (49) in mineral salts medium at 30°C lithoautotrophically in an atmosphere of H_2–CO_2–O_2 (8:1:1; vol/vol/vol), organoautotrophically with 0.2% (wt/vol) formate, or heterotrophically with 0.2% (wt/vol) organic substrate. *E. coli* strains were cultured in LB medium (38) at 37°C. The media contained antibiotics, when needed, at the following concentrations: 50 μg of ampicillin per ml or 350 μg of kanamycin per ml. For gene expression experiments, *E. coli* strains harboring various pUC-derived plasmids were grown in LB medium containing ampicillin. When an optical density at 550 nm of 0.5 was reached, the *lac* promoter was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubation was continued for an additional 4 h.

Preparation of cell extracts. Cells to be used for the preparation of extracts were harvested in the exponential growth phase, washed, and resuspended in ice-cold buffer (20 mM Tris-HCl [pH 7.6] containing 10 mM $MgCl_2$ and 1 mM dithioerythritol) at a density of about 20 mg of cell protein per ml. Cell disruption was achieved by either sonication (*E. coli*) or French press treatment (*A. eutrophus*). The supernatant resulting from a subsequent centrifugation of the homogenate at 100,000 × *g* for 1 h was used directly for assays. Protein concentrations were estimated by the method of Lowry et al. (32).

For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), cells from 1-ml culture samples were harvested by centrifugation and resuspended in 100 μl of SDS cracking buffer (50 mM Tris-HCl [pH 6.8], 100 mM dithioerythritol, 2% [wt/vol] SDS, 0.1% [wt/vol] bromophenol blue, 10% [wt/vol] glycerol). After boiling for 3 to 5 min, 10-μl aliquots of the lysates were subjected to SDS-PAGE according to Laemmli (28). Silver staining (5) was used to visualize proteins in gels.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant phenotype or genotype[a]	Source or reference
Strains		
A. eutrophus		
H16	Cfx^+ Hox^+ $pHG1^+$	ATCC 17699
HB9	Sm^r Km^r Cfx^- Hox^- $pHG1^-$; chromosomal *cbb* operon inactivated by a Tn*5* insertion	49
HB9(pHG1)	Sm^r Km^r Cfx^+ Hox^+ $pHG1^+$	49
E. coli		
JW1	*ara* Δ(*lac-proAB*) *rpsL* φ80 *lacZΔM15* F′ *lacI*^{q}*ZΔM15 proA*$^+$*B*$^+$	25
JM109	*endA1 recA1 syrA96 thi hsdR17* (r_K^- m_D^+) *relA1 supE44* λ$^-$ Δ(*lac-proAB*) F′ *traD36 proAB lacI*^{q}*ZΔM15*	52
Plasmids[b]		
pUC18	Ap^r *lacPOZ′*	46
pUC19	Ap^r *lacPOZ′*	46
pAEC8010	pUC18::2.9-kb *Bam*HI	This study[c]
pAEC8011	pUC18::2.9-kb *Bam*HI	This study
pAEC4300	pUC19::1.4-kb *Ava*II-*Eco*RV (in *Sma*I)	This study
pAEC4301	pUC19::1.4-kb *Ava*II-*Eco*RV (in *Sma*I)	This study
pAEP8230	pUC18::2.4-kb *Sph*I	This study
pAEP8231	pUC18::2.4-kb *Sph*I	This study
pAEP4310	pUC19::2.3-kb *Eco*RV (in *Sma*I)	This study
pAEP4320	pUC19::1.3-kb *Eco*RI-*Xho*I[d]	This study

[a] Ap^r, ampicillin resistant; Km^r, kanamycin resistant; Sm^r, streptomycin resistant; Cfx, ability to fix CO_2 autotrophically; Hox, ability to oxidize H_2; pHG1, megaplasmid pHG1 of *A. eutrophus* H16 (15).
[b] The last digit of the pAE plasmid designations indicates the orientation of the subcloned *cbb* genes relative to the *lac* promoter (0, collinear; 1, divergent).
[c] See Fig. 1.
[d] See Materials and Methods.

Enzyme assays. PGP activity was determined by measuring the amount of P_i released upon hydrolysis of 2-phosphoglycolate at 30°C. The assay was run with a 0.3-ml reaction mixture containing 40 mM morpholinepropanesulfonic acid (MOPS)-NaOH (pH 7.0)–10 mM $MgCl_2$, 4 mM 2-phosphoglycolate, and cell extract. Following its start by the addition of 2-phosphoglycolate, the reaction was stopped after an appropriate time by the addition of 60 μl of 2.5 N H_2SO_4. The amount of liberated P_i was estimated colorimetrically as described by Ames (2).

TK activity was assayed in a coupled optical test by monitoring the oxidation of NADH at 340 nm and 30°C in a 0.6-ml reaction mixture containing 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 1 mM thiamine diphosphate, 2 mM xylulose-5-phosphate, 2 mM ribose-5-phosphate, 0.2 mM NADH, 3 U of α-glycerophosphate dehydrogenase, 9 U of triosephosphate isomerase, and cell extract. Enzyme activities are given in international units. One unit is defined as the amount of enzyme that converts 1 μmol of substrate per min into product(s).

Gel filtration. After the removal of insoluble material by centrifugation and an additional passage through a membrane filter with a 0.2-μm-pore size, cell extracts of *E. coli* (pAEC4300) or lithoautotrophically grown *A. eutrophus* H16 were chromatographed on a Sephacryl S-300 HR column (1.6 by 87 cm; bed volume, 175 ml) that had been equilibrated with 50 mM Tris-HCl (pH 7.5) buffer containing 10 mM $MgCl_2$ and 1 mM dithioerythritol. The PGP activity in the fractions (1 ml) was estimated as described above. To obtain the native M_r of PGP, the column was calibrated with proteins having known molecular masses (catalase [240 kDa], bovine serum albumin [136 or 68 kDa], and carbonic anhydrase [12.5 kDa]).

DNA techniques and sequence analysis. The following procedures were carried out by standard methods (3, 38): preparation of plasmid DNA, agarose gel electrophoresis, DNA ligation, and bacterial transformation. Enzymes were used under the conditions recommended by the commercial suppliers. DNA fragments were extracted from agarose gels by the procedure described by Weichenhan (47). Sequencing was done on double-stranded plasmid DNA by the dideoxy chain termination method (39). To reduce the formation of secondary structures in the polynucleotide products, dGTP was substituted for by 7-deaza-dGTP. Both strands of the DNA fragment in question were completely sequenced by use of appropriately generated deletion clones (16) and/or sequence-specific oligodeoxynucleotide primers (17-mers) synthesized on a Gene Assembler Plus DNA synthesizer (Pharmacia, Freiburg, Germany). Nucleotide and derived amino acid sequences were analyzed by the latest available versions of the GENMON programs (GBF, Braunschweig, Germany) and the GCG program package of the University of Wisconsin (12), including similarity searches of the GenBank (Los Alamos National Laboratory, Los Alamos, N.Mex.), EMBL/SwissProt (Heidelberg, Germany), and PIR (Georgetown University Medical Center, Washington, D.C.) data bases by use of the TFASTA (35) and BLAST (1) programs.

Construction of plasmids. Plasmid vectors (pUC18 and pUC19) were digested to completion with restriction endonucleases and dephosphorylated by treatment with alkaline phosphatase. DNA fragments derived from recombinant phages λAEC2 and λAEP2 (17) and containing the chromosomal and plasmid-encoded *cbb* genes, respectively, were used for subcloning. The fragments (Table 1 and Fig. 1) were blunt ended, if necessary, by filling 5′-protruding ends with the aid of the Klenow fragment of DNA polymerase I, ligated to vector DNA, and eventually transformed into *E. coli* strains. For construction of plasmid pAEP4320, DNA of pAEP4310 was digested with *Eco*RI and *Xho*I, and the resulting 1.3-kb fragment was subsequently ligated to pUC19 that had been cleaved with *Eco*RI and *Sal*I.

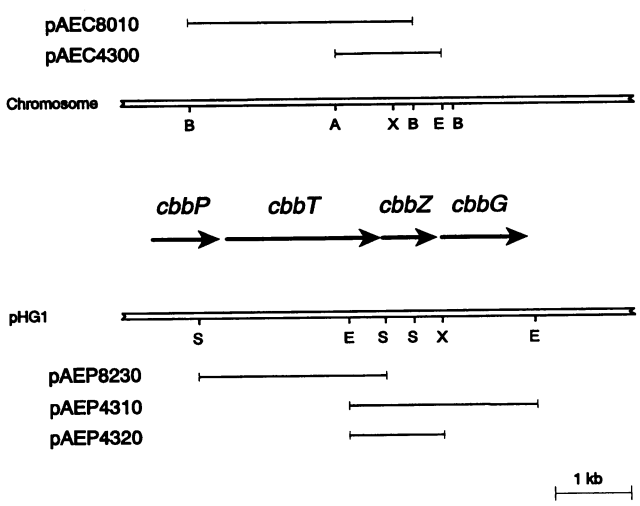


FIG. 1. Physical and genetic maps of the analyzed regions from the two *cbb* operons of *A. eutrophus* H16. The genes, with their relative orientations and sizes, are indicated by arrows. Various subfragments cloned into expression vectors pUC18 and pUC19 are shown above (chromosome) and below (megaplasmid pHG1) the maps, together with the corresponding designations of the hybrid plasmids. Gene designations: *cbbP*, gene encoding phosphoribulokinase; *cbbT*, TK gene; *cbbZ*, PGP gene; *cbbG*, glyceraldehyde-3-phosphate dehydrogenase gene. Abbreviations for cleavage sites of restriction enzymes: A, *Ava*II; B, *Bam*HI; E, *Eco*RV; S, *Sph*I; X, *Xho*I.

Enzymes and chemicals. Restriction enzymes, T4 DNA ligase, and alkaline phosphatase were obtained from GIBCO-BRL (Eggenstein, Germany), Boehringer GmbH (Mannheim, Germany), or Pharmacia and used as recommended by the manufacturers. Pharmacia was also the supplier of the Klenow fragment of DNA polymerase I, nucleotides and chemicals used for oligodeoxynucleotide synthesis, and the Sephacryl S-300 HR column. Antibiotics, ribose-5-phosphate, NADH, triosephosphate isomerase, and reference proteins for SDS-PAGE and gel filtration experiments were purchased from Boehringer. Sigma Chemie (Deisenhofen, Germany) provided 2-phosphoglycolate, thiamine diphosphate, and xylulose-5-phosphate.

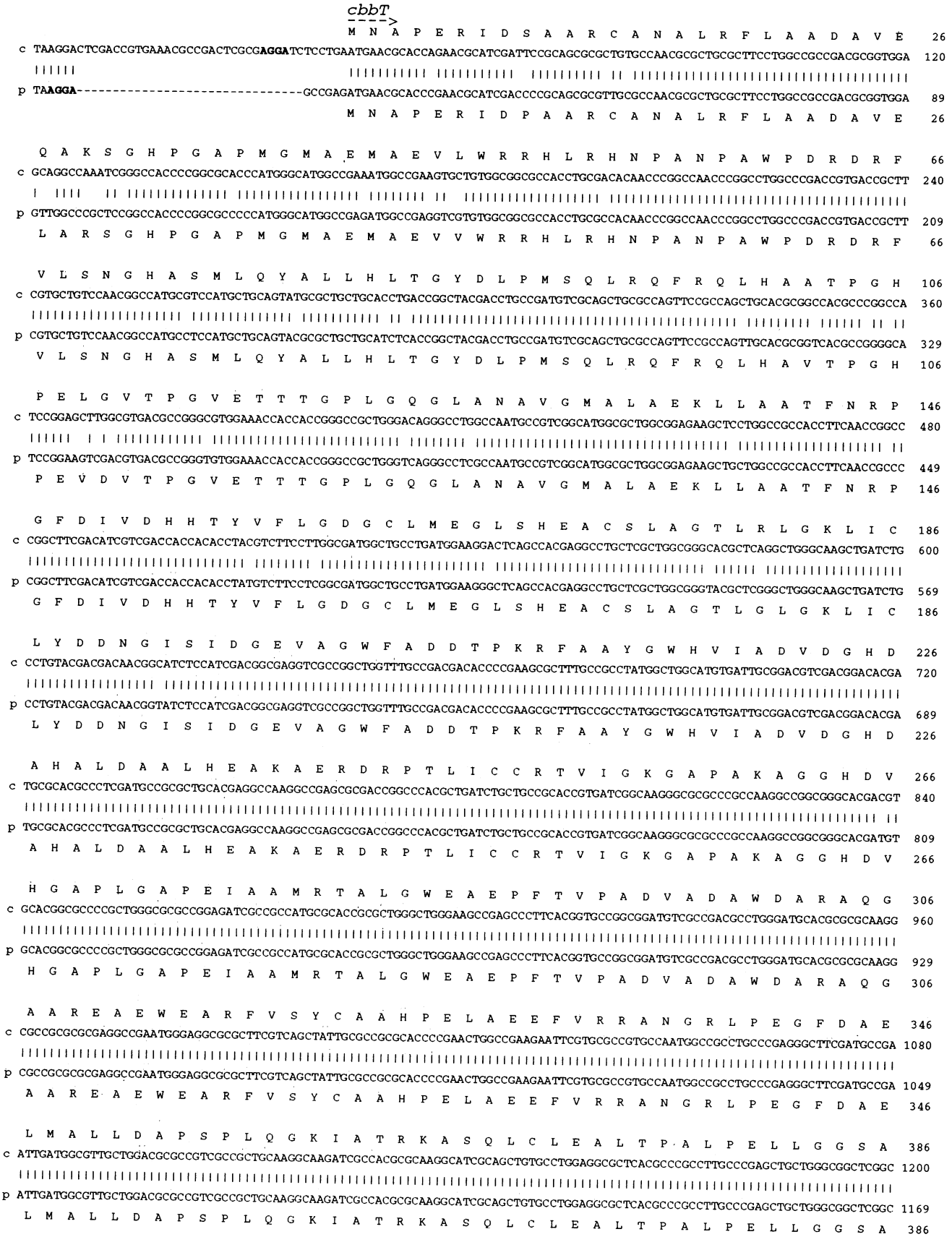
Nucleotide sequence accession numbers. The nucleotide sequences have been deposited in the GenBank data base under accession numbers M68904 (chromosomal sequence) and M68905 (plasmid-encoded sequence).

RESULTS

Sequence analysis of the regions between *cbbP* and *cbbG*. The regions between the previously identified genes *cbbP* (23, 24, 26) and *cbbG* (48) were sequenced from both the chromosomal and the pHG1-encoded *cbb* operons. Within these regions, two additional closely linked open reading frames, designated *cbbT* and *cbbZ* (Fig. 2), were found to be oriented in the same direction as the other genes of the operon. The 5′ end of *cbbT* had been detected in an earlier study (26). Because of a 31-bp deletion in the intergenic region, the plasmid-encoded *cbbP* and *cbbT* genes are more tightly linked than the two chromosomal copies. The start codons of both chromosomal (c) and plasmid-encoded (p) *cbbT* and *cbbZ* are separated from the corresponding putative ribosome-binding sequences by the usual distances observed in *A. eutrophus* (20, 26, 40, 50) and *E. coli* (41). The stop codons of $cbbT_{c/p}$ and the start codons of $cbbZ_{c/p}$ even overlap by 1 bp. No further potential open reading frame of significant size or codon bias was detected in either orientation of the analyzed regions. The chromosomal and plasmid-encoded sequences showed the expected very high overall (nucleotide level and amino acid level) identities of 96 and 97% (*cbbT*) or 93 and 94% (*cbbZ*) typical for the duplicated *cbb* gene clusters. The calculated properties of the genes and their potential products are listed in Table 2. Comparisons of the deduced amino acid sequences with sequences in several data libraries revealed striking similarities of CbbT to the TK from the phototrophic bacterium *Rhodobacter sphaeroides* (9), the yeast *Saccharomyces cerevisiae* (14), and *E. coli* (43); the dihydroxyacetone synthase from the methylotrophic yeast *Hansenula polymorpha* (19); and the RecP protein from *Streptococcus pneumoniae* (36). No significant overall resemblance to any reported protein sequence was detected for CbbZ, but there were some partial similarities to various gene products (see Discussion).

Heterologous expression of the *cbb* genes. The identities and enzyme activities of the *cbb* gene products encoded on recombinant plasmids were verified by heterologous gene expression. For this purpose, plasmids carrying suitable subfragments cloned in expression vectors pUC18 and pUC19 (Table 1 and Fig. 1) were transformed into *E. coli* JW1 or JM109, and the *cbb* genes were expressed in the foreign host under the control of the *lac* promoter. The molecular masses of overproduced proteins CbbT (74 kDa) and CbbZ (24 kDa) detected by SDS-PAGE (Fig. 3) corresponded well to those estimated from the deduced amino acid sequences. In all cases, overexpression of the *cbb* genes depended on the *lac* promoter, indicating that the genes do not have a promoter that is functional in the *E. coli* host. Significantly enhanced TK activities were found in strains containing the *cbbT* genes, but they exceeded the background level in *E. coli* by 2.6- to 6.4-fold only in strains harboring the *cbbT* genes in a collinear orientation relative to the *lac* promoter on the expression vectors (Table 3). The pHG1-encoded *cbbT* gene was apparently less strongly expressed than the chromosomal copy, correlating with the amounts of overproduced TK proteins in the strains (Fig. 3). The level of TK activity in *A. eutrophus*, supposed to reflect the rate of synthesis of the enzyme, was high in autotrophically grown cells, whereas heterotrophic growth on fructose led to an activity level about twice that found in pyruvate-grown cells (Table 3). Pyruvate is known to completely repress the *cbb* operons, while fructose causes partial derepression (29).

E. coli is not known to contain PGP, as was confirmed by the lack of PGP activity in reference clone *E. coli*(pUC18). However, a very high level of PGP activity was present in transconjugant *E. coli*(pAEC4300). This finding is regarded to be unambiguous evidence that *cbbZ* encodes the PGP of *A. eutrophus*, although the plasmid-encoded *cbbZ* gene was apparently not expressed in *E. coli*(pAEP4320) (Table 4). The gene did not lead to detectable overproduction of a protein (Fig. 3), and in a clone harboring $cbbZ_p$ together with $cbbG_p$ [*E. coli*(pAEP4310)], only the product of the latter gene was found to be overproduced. Moreover, no PGP activity or overproduced protein was obtained with an alternative $cbbZ_p$ clone (data not shown). Lithoautotrophically grown wild-type *A. eutrophus* H16 exhibited significant PGP activity. In contrast, pyruvate-grown cells contained no detectable activity, like mutant HB9, a pHG1-free strain with the chromosomal *cbb* operon inactivated by Tn*5* insertion (49), after growth on fructose plus formate (Table 4). Even lithoautotrophically grown transconjugant HB9(pHG1) showed no PGP activity, providing evidence that only the chromosomal *cbbZ* gene is


cbbT
M N A P E R I D S A A R C A N A L R F L A A D A V E 26
c TAAGGACTCGACCGTGAAACGCCGACTCGCGAGGATCTCCTGAATGAACGCACCAGAACGCATCGATTCCGCAGCGCGCTGTGCCAACGCGCTGCGCTTCCTGGCCGCCGACGCGGTGGA 120
p TAAGGA----------GCCGAGATGAACGCACCCGAACGCATCGACCCCGCAGCGCGTTGCGCCAACGCGCTGCGCTTCCTGGCCGCCGACGCGGTGGA 89
M N A P E R I D P A A R C A N A L R F L A A D A V E 26
Q A K S G H P G A P M G M A E M A E V L W R R H L R H N P A N P A W P D R D R F 66
c GCAGGCCAAATCGGGCCACCCCGGCGCACCCATGGGCATGGCCGAAATGGCCGAAGTGCTGTGGCGGCGCCACCTGCGACACAACCCGGCCAACCCGGCCTGGCCCGACCGTGACCGCTT 240
p GTTGGCCCGCTCCGGCCACCCCGGCGCCCCCATGGGCATGGCCGAGATGGCCGAGGTCGTGTGGCGGCGCCACCTGCGCCACAACCCGGCCAACCCGGCCTGGCCCGACCGTGACCGCTT 209
L A R S G H P G A P M G M A E M A E V V W R R H L R H N P A N P A W P D R D R F 66
V L S N G H A S M L Q Y A L L H L T G Y D L P M S Q L R Q F R Q L H A A T P G H 106
c CGTGCTGTCCAACGGCCATGCGTCCATGCTGCAGTATGCGCTGCTGCACCTGACCGGCTACGACCTGCCGATGTCGCAGCTGCGCCAGTTCCGCCAGCTGCACGCGGCCACGCCCGGCCA 360
p CGTGCTGTCCAACGGCCATGCCTCCATGCTGCAGTACGCGCTGCTGCATCTCACCGGCTACGACCTGCCGATGTCGCAGCTGCGCCAGTTCCGCCAGTTGCACGCGGTCACGCCGGGGCA 329
V L S N G H A S M L Q Y A L L H L T G Y D L P M S Q L R Q F R Q L H A V T P G H 106
P E L G V T P G V E T T T G P L G Q G L A N A V G M A L A E K L L A A T F N R P 146
c TCCGGAGCTTGGCGTGACGCCGGGCGTGGAAACCACCACCGGGCCGCTGGGACAGGGCCTGGCCAATGCCGTCGGCATGGCGCTGGCGGAGAAGCTCCTGGCCGCCACCTTCAACCGGCC 480
p TCCGGAAGTCGACGTGACGCCGGGTGTGGAAACCACCACCGGGCCGCTGGGTCAGGGCCTCGCCAATGCCGTCGGCATGGCGCTGGCGGAGAAGCTGCTGGCCGCCACCTTCAACCGCCC 449
P E V D V T P G V E T T T G P L G Q G L A N A V G M A L A E K L L A A T F N R P 146
G F D I V D H H T Y V F L G D G C L M E G L S H E A C S L A G T L R L G K L I C 186
c CGGCTTCGACATCGTCGACCACCACACCTACGTCTTCCTTGGCGATGGCTGCCTGATGGAAGGACTCAGCCACGAGGCCTGCTCGCTGGCGGGCACGCTCAGGCTGGGCAAGCTGATCTG 600
p CGGCTTCGACATCGTCGACCACCACACCTATGTCTTCCTCGGCGATGGCTGCCTGATGGAAGGGCTCAGCCACGAGGCCTGCTCGCTGGCGGGTACGCTCGGGCTGGGCAAGCTGATCTG 569
G F D I V D H H T Y V F L G D G C L M E G L S H E A C S L A G T L G L G K L I C 186
L Y D D N G I S I D G E V A G W F A D D T P K R F A A Y G W H V I A D V D G H D 226
c CCTGTACGACGACAACGGCATCTCCATCGACGGCGAGGTCGCCGGCTGGTTTGCCGACGACACCCCGAAGCGCTTTGCCGCCTATGGCTGGCATGTGATTGCGGACGTCGACGGACACGA 720
p CCTGTACGACGACAACGGTATCTCCATCGACGGCGAGGTCGCCGGCTGGTTTGCCGACGACACCCCGAAGCGCTTTGCCGCCTATGGCTGGCATGTGATTGCGGACGTCGACGGACACGA 689
L Y D D N G I S I D G E V A G W F A D D T P K R F A A Y G W H V I A D V D G H D 226
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c TGCGCACGCCCTCGATGCCGCGCTGCACGAGGCCAAGGCCGAGCGCGACCGGCCCACGCTGATCTGCTGCCGCACCGTGATCGGCAAGGGCGCGCCCGCCAAGGCCGGCGGGCACGACGT 840
p TGCGCACGCCCTCGATGCCGCGCTGCACGAGGCCAAGGCCGAGCGCGACCGGCCCACGCTGATCTGCTGCCGCACCGTGATCGGCAAGGGCGCGCCCGCCAAGGCCGGCGGGCACGATGT 809
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c GCACGGCGCCCCGCTGGGCGCGCCGGAGATCGCCGCCATGCGCACCGCGCTGGGCTGGGAAGCCGAGCCCTTCACGGTGCCGGCGGATGTCGCCGACGCCTGGGATGCACGCGCGCAAGG 960
p GCACGGCGCCCCGCTGGGCGCGCCGGAGATCGCCGCCATGCGCACCGCGCTGGGCTGGGAAGCCGAGCCCTTCACGGTGCCGGCGGATGTCGCCGACGCCTGGGATGCACGCGCGCAAGG 929
H G A P L G A P E I A A M R T A L G W E A E P F T V P A D V A D A W D A R A Q G 306
A A R E A E W E A R F V S Y C A A H P E L A E E F V R R A N G R L P E G F D A E 346
c CGCCGCGCGCGAGGCCGAATGGGAGGCGCGCTTCGTCAGCTATTGCGCCGCGCACCCCGAACTGGCCGAAGAATTCGTGCGCCGTGCCAATGGCCGCCTGCCCGAGGGCTTCGATGCCGA 1080
p CGCCGCGCGCGAGGCCGAATGGGAGGCGCGCTTCGTCAGCTATTGCGCCGCGCACCCCGAACTGGCCGAAGAATTCGTGCGCCGTGCCAATGGCCGCCTGCCCGAGGGCTTCGATGCCGA 1049
A A R E A E W E A R F V S Y C A A H P E L A E E F V R R A N G R L P E G F D A E 346
L M A L L D A P S P L Q G K I A T R K A S Q L C L E A L T P A L P E L L G G S A 386
c ATTGATGGCGTTGCTGGACGCGCCGTCGCCGCTGCAAGGCAAGATCGCCACGCGCAAGGCATCGCAGCTGTGCCTGGAGGCGCTCACGCCCGCCTTGCCCGAGCTGCTGGGCGGCTCGGC 1200
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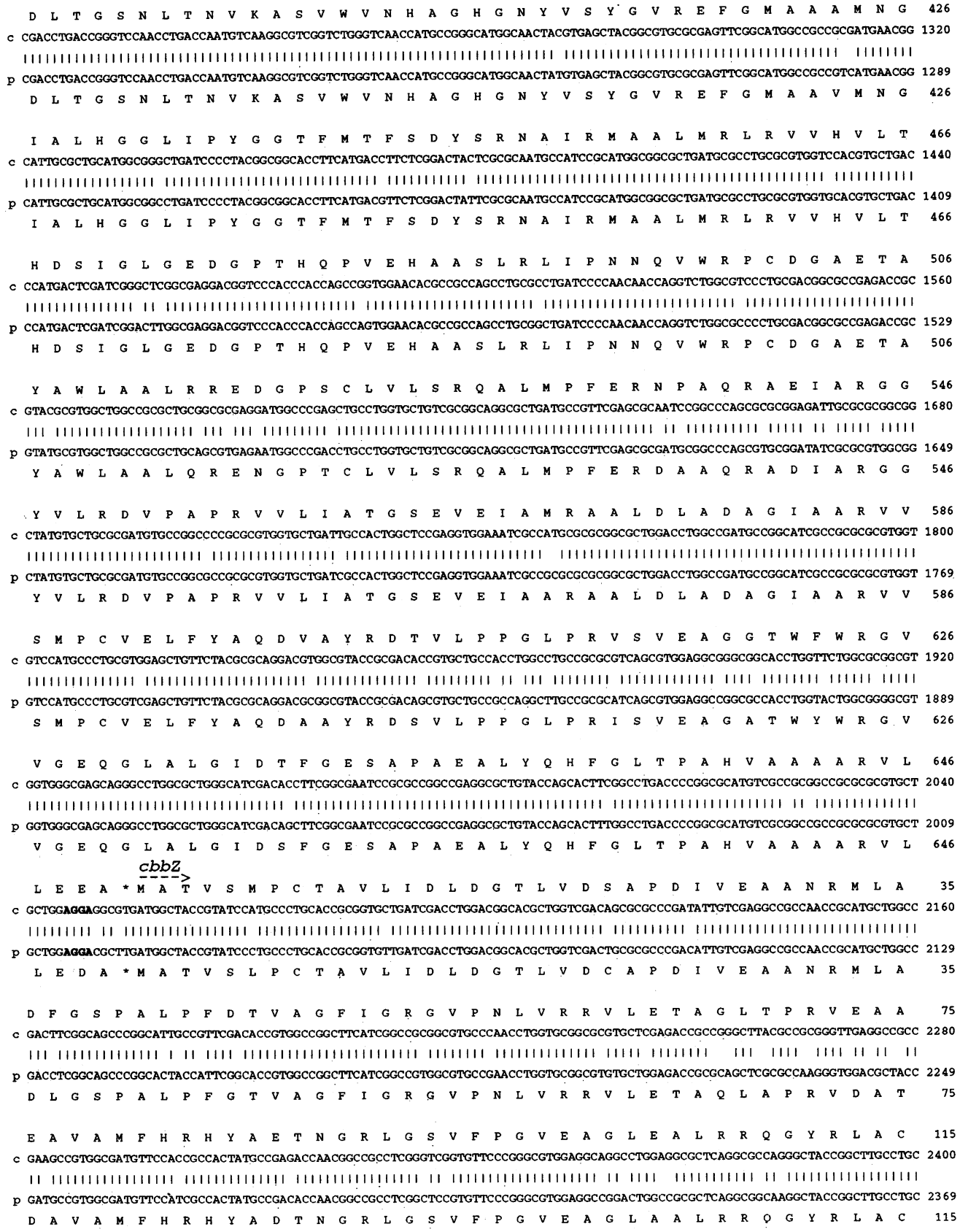
FIG. 2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D L T G S N L T N V K A S V W V N H A G H G N Y V S Y G V R E F G M A A V M N G 426
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p CATTGCGCTGCATGGCGGCCTGATCCCCTACGGCGGCACCTTCATGACGTTCTCGGACTATTCGCGCAATGCCATCCGCATGGCGGCGCTGATGCGCCTGCGCGTGGTGCACGTGCTGAC 1409
I A L H G G L I P Y G G T F M T F S D Y S R N A I R M A A L M R L R V V H V L T 466
H D S I G L G E D G P T H Q P V E H A A S L R L I P N N Q V W R P C D G A E T A 506
c CCATGACTCGATCGGGCTCGGCGAGGACGGTCCCACCCACCAGCCGGTGGAACACGCCGCCAGCCTGCGCCTGATCCCCAACAACCAGGTCTGGCGTCCCTGCGACGGCGCCGAGACCGC 1560
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p GTATGCGTGGCTGGCCGCGCTGCAGCGTGAGAATGGCCCGACCTGCCTGGTGCTGTCGCGGCAGGCGCTGATGCCGTTCGAGCGCGATGCGGCCCAGCGTGCGGATATCGCGCGTGGCGG 1649
Y A W L A A L Q R E N G P T C L V L S R Q A L M P F E R D A A Q R A D I A R G G 546
Y V L R D V P A P R V V L I A T G S E V E I A M R A A L D L A D A G I A A R V V 586
c CTATGTGCTGCGCGATGTGCCGGCCCCGCGCGTGGTGCTGATTGCCACTGGCTCCGAGGTGGAAATCGCCATGCGCGCGGCGCTGGACCTGGCCGATGCCGGCATCGCCGCGCGCGTGGT 1800
p CTATGTGCTGCGCGATGTGCCGGCGCCGCGCGTGGTGCTGATCGCCACTGGCTCCGAGGTGGAAATCGCCGCGCGCGCGGCGCTGGACCTGGCCGATGCCGGCATCGCCGCGCGCGTGGT 1769
Y V L R D V P A P R V V L I A T G S E V E I A A R A A L D L A D A G I A A R V V 586
S M P C V E L F Y A Q D V A Y R D T V L P P G L P R V S V E A G G T W F W R G V 626
c GTCCATGCCCTGCGTGGAGCTGTTCTACGCGCAGGACGTGGCGTACCGCGACACCGTGCTGCCACCTGGCCTGCCGCGCGTCAGCGTGGAGGCGGGCGGCACCTGGTTCTGGCGCGGCGT 1920
p GTCCATGCCCTGCGTCGAGCTGTTCTACGCGCAGGACGCGGCGTACCGCGACAGCGTGCTGCCGCCAGGCTTGCCGCGCATCAGCGTGGAGGCCGGCGCCACCTGGTACTGGCGGGGCGT 1889
S M P C V E L F Y A Q D A A Y R D S V L P P G L P R I S V E A G A T W Y W R G V 626
V G E Q G L A L G I D T F G E S A P A E A L Y Q H F G L T P A H V A A A A R V L 646
c GGTGGGCGAGCAGGGCCTGGCGCTGGGCATCGACACCTTCGGCGAATCCGCGCCGGCCGAGGCGCTGTACCAGCACTTCGGCCTGACCCCGGCGCATGTCGCCGCGGCCGCGCGCGTGCT 2040
p GGTGGGCGAGCAGGGCCTGGCGCTGGGCATCGACAGCTTCGGCGAATCCGCGCCGGCCGAGGCGCTGTACCAGCACTTTGGCCTGACCCCGGCGCATGTCGCGGCCGCCGCGCGCGTGCT 2009
V G E Q G L A L G I D S F G E S A P A E A L Y Q H F G L T P A H V A A A A R V L 646
cbbZ
L E E A *M A T V S M P C T A V L I D L D G T L V D S A P D I V E A A N R M L A 35
c GCTGGAGGAGGCGTGATGGCTACCGTATCCATGCCCTGCACCGCGGTGCTGATCGACCTGGACGGCACGCTGGTCGACAGCGCGCCCGATATTGTCGAGGCCGCCAACCGCATGCTGGCC 2160
p GCTGGAGGACGCTTGATGGCTACCGTATCCCTGCCCTGCACCGCGGTGTTGATCGACCTGGACGGCACGCTGGTCGACTGCGCGCCCGACATTGTCGAGGCCGCCAACCGCATGCTGGCC 2129
L E D A *M A T V S L P C T A V L I D L D G T L V D C A P D I V E A A N R M L A 35
D F G S P A L P F D T V A G F I G R G V P N L V R R V L E T A G L T P R V E A A 75
c GACTTCGGCAGCCCGGCATTGCCGTTCGACACCGTGGCCGGCTTCATCGGCCGCGGCGTGCCCAACCTGGTGCGGCGCGTGCTCGAGACCGCCGGGCTTACGCCGCGGGTTGAGGCCGCC 2280
p GACCTCGGCAGCCCGGCACTACCATTCGGCACCGTGGCCGGCTTCATCGGCCGTGGCGTGCCGAACCTGGTGCGGCGTGTGCTGGAGACCGCGCAGCTCGCGCCAAGGGTGGACGCTACC 2249
D L G S P A L P F G T V A G F I G R G V P N L V R R V L E T A Q L A P R V D A T 75
E A V A M F H R H Y A E T N G R L G S V F P G V E A G L E A L R R Q G Y R L A C 115
c GAAGCCGTGGCGATGTTCCACCGCCACTATGCCGAGACCAACGGCCGCCTCGGGTCGGTGTTCCCGGGCGTGGAGGCAGGCCTGGAGGCGCTCAGGCGCCAGGGCTACCGGCTTGCCTGC 2400
p GATGCCGTGGCGATGTTCCATCGCCACTATGCCGACACCAACGGCCGCCTCGGCTCCGTGTTCCCGGGCGTGGAGGCCGGACTGGCCGCGCTCAGGCGGCAAGGCTACCGGCTTGCCTGC 2369
D A V A M F H R H Y A D T N G R L G S V F P G V E A G L A A L R R Q G Y R L A C 115

FIG. 2

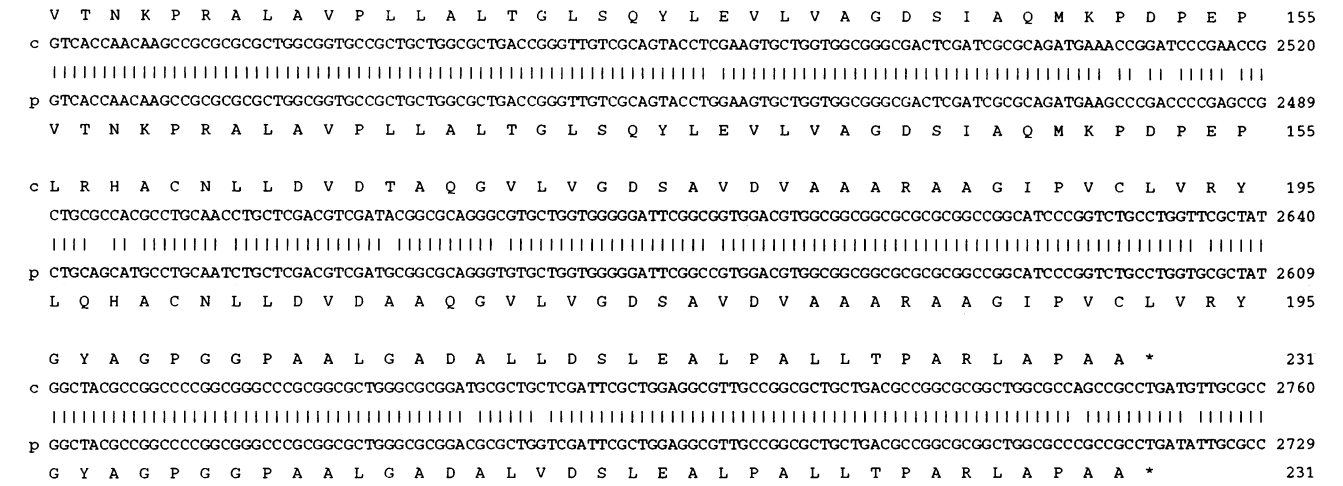

FIG. 2. Aligned nucleotide and deduced amino acid sequences of the analyzed regions from the chromosomal (c) and pHG1-encoded (p) *cbb* operons of *A. eutrophus* H16. Amino acids are indicated in the one-letter code above or below the respective codons. The putative ribosome-binding sites are printed in boldface type. Gaps (dashes) were introduced to optimize the sequence alignment. Vertical lines indicate identical bases, and asterisks indicate stop codons. The nucleotide sequences overlap with those reported earlier (26) by 274 (c) and 243 (p) positions at their 5′ ends.

functionally expressed in *A. eutrophus*, as in the heterologous host *E. coli*.

Molecular weight of native PGP. To gain information on the structure of native PGP from *A. eutrophus*, gel filtration experiments were performed with a Sephacryl S-300 HR column. Analyses of cell extracts from both recombinant *E. coli*(pAEC4300) and the authentic host *A. eutrophus* H16 yielded an M_r of 70,500 for native PGP (data not shown), compatible with a homotrimeric structure of the enzyme.

DISCUSSION

The data reported provided genetic and enzymatic evidence for two additional structural genes, *cbbT* and *cbbZ*, which are encoded within the *cbb* operons of *A. eutrophus* H16. Sequence analysis and heterologous expression showed that the products of both genes, TK and PGP, respectively, function as enzymes in the Calvin cycle (TK) or in the glycolate metabolism (PGP) of this chemoautotroph. As indicated by the corresponding enzyme activities, the expression of both genes in *A. eutrophus* follows a regulatory pattern similar to that observed for other enzymes of the Calvin cycle (7). This finding, together with the requirement for the *lac* promoter for *cbbT* and *cbbZ* expression in *E. coli*, supports the conclusion that the two contiguous genes are part of the large *cbb* operon, including also the 3′-flanking *cbbG*. Thus, as already shown for RubisCO (17), pentose-5-phosphate 3-epimerase (27), phosphoribulokinase (23), and glyceraldehyde-3-phosphate dehydrogenase (48), *A. eutrophus* forms two TK isoenzymes operating in the Calvin cycle. The dual involvement of TK in general pentose metabolism and in autotrophic CO_2 fixation has important metabolic consequences for the facultatively autotrophic organism. Since the *cbb* operons are completely repressed during growth on various organic acids and mutants with defective *cbb* operons are unaffected in heterotrophic growth (49), the organism must be able to form a third TK isoenzyme functioning in heterotrophic carbon metabolism. This additional isoenzyme is probably represented by the low TK activity in cells grown on pyruvate, which fully represses the *cbb* operons.

TABLE 2. Calculated characteristics of the identified genes and their products

Gene or protein	Length (bp)	G+C content (mol %)	No. of amino acids	Molecular mass (kDa)	pI
Genes					
cbbT					
Chromosome	2,013	70.54			
Plasmid	2,013	70.79			
cbbZ					
Chromosome	696	72.56			
Plasmid	696	72.56			
Proteins					
CbbT					
Chromosome			670	71.6	5.90
Plasmid			670	71.5	5.65
CbbZ					
Chromosome			231	23.8	4.77
Plasmid			231	23.6	4.82

TK catalyzes the transfer of the two-carbon glycolaldehyde group from ketoses to aldoses with a relatively broad substrate specificity. Recently, the three-dimensional structure of TK from *S. cerevisiae* was determined (30), allowing deeper insights into the reaction mechanism and identifying the amino acid residues critical for cofactor binding. It has been shown that the enzyme is composed of two identical 75-kDa subunits, contains one molecule each of Ca^{2+} and thiamine diphosphate, and requires Mg^{2+} for catalytic activity (8, 30). Sequence alignments (Fig. 4) with TK from *S. cerevisiae*, *R. sphaeroides*, and *E. coli*, with dihydroxyacetone synthase from *H. polymorpha*, a novel type of TK, and the RecP protein from *S. pneumoniae*, which might be a TK, confirmed that CbbT from *A. eutrophus* is a TK protein. The primary structures of the enzymes are relatively conserved, showing identities to CbbT of between 35 and 51%. Particularly, amino acid residues essential for thiamine diphosphate binding (His-79, Gly-

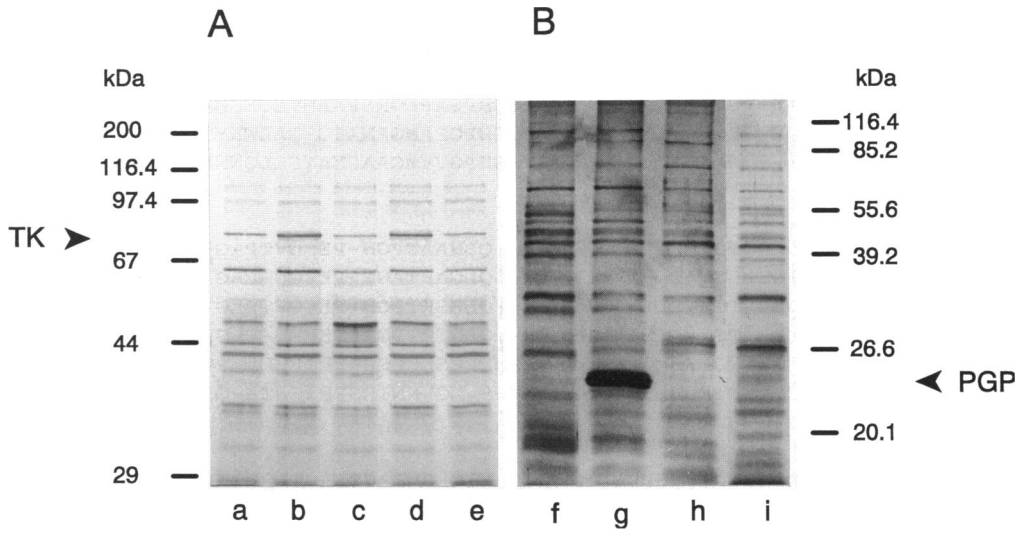

FIG. 3. Heterologous expression of both chromosomal and plasmid-encoded *cbbT* (A) and *cbbZ* (B) genes in transformants of *E. coli*. Shown is SDS-PAGE of lysed *E. coli* JM109 containing pUC18 (lane a), pAEC8010 (lane b), pAEC8011 (lane c), pAEP8230 (lane d), or pAEP8231 (lane e) and of lysed *E. coli* JW1 containing pUC18 (lane f), pAEC4300 (lane g), pAEC4301 (lane h), or pAEP4320 (lane i). Cells were grown in LB medium supplemented with ampicillin, and the *lac* promoter on the plasmids was induced with 0.5 mM IPTG. Protein (about 1 [A] or 2 [B] μg per lane) was visualized by silver staining. The overproduced TK and PGP proteins are marked by arrowheads. Numbers indicate the sizes of molecular mass markers in kilodaltons.

116, Leu-118, Gly-159, Glu-163, Asp-186, Ile-192, His-264, Asp-383*, Leu-384*, Ile-418*, Glu-424*, Phe-448*, Phe-451*, Tyr-454*, and His-487* [the asterisk indicates a residue from the second subunit]), Ca^{2+} binding (Asp-158, Asn-188, and Ile-190), and substrate binding (Arg-94, Glu-482*, and Asp-483*) (30) are identical or conservatively exchanged in all cases, with the exception of RecP, for which the equivalents of Asp-186 and Asn-188 are missing (Fig. 4). The histidine residues His-30, His-103, and His-487*, which might be of importance in proton transfer during catalysis and in stabilization of the covalent intermediate between the substrate and thiamine diphosphate (30), are also conserved in the TK examined. The determined molecular mass of about 72 kDa for the subunits of the enzyme from *A. eutrophus* is comparable to that of other TK subunits, e.g., 69 kDa for the subunits of the enzyme from *R. sphaeroides*.

To our knowledge, *cbbZ* is the first PGP gene that has been cloned from any organism. The placement of this gene within the *cbb* operons is of particular interest. It might be considered the genetic manifestation of the usual metabolic situation in autotrophically growing *A. eutrophus* in the natural habitat. The formation of 2-phosphoglycolate by the action of bifunctional RubisCO in the presence of relatively low CO_2 and high O_2 concentrations would lead to an accumulation of the compound as a dead-end inhibitory metabolite (inhibition of triosephosphate isomerase [51]), unless it were dephosphorylated by PGP to yield glycolate. Further breakdown of glycolate would allow the major part of the carbon fraction flowing into 2-phosphoglycolate to be recycled into the Calvin cycle. Photorespiration in plants (34) is based on this carbon-salvaging glycolate metabolism. *A. eutrophus* has been observed to excrete glycolate under certain conditions (11, 21), confirming the physiological role of PGP. The coordinated synthesis of RubisCO and PGP achieved because of their being encoded within a common operon is probably favorable for keeping the critical metabolic balance at the interface of two interlocking pathways. It was surprising to find that, despite the very high homology between chromosomal and plasmid-encoded *cbbZ*

TABLE 3. Activities of TK in cell extracts of *A. eutrophus* grown with various substrates and in transformant strains of *E. coli*

Strain[a]	Growth substrate	Sp act (U/mg of protein)
A. eutrophus H16	H_2-CO_2[b]	0.152
A. eutrophus H16	Formate[c]	0.256
A. eutrophus H16	Fructose	0.029
A. eutrophus H16	Pyruvate	0.015
E. coli(pAEC8010)	LB[d]	1.307
E. coli(pAEC8011)	LB	0.194
E. coli(pAEP8230)	LB	0.536
E. coli(pAEP8231)	LB	0.205
E. coli(pUC18)	LB	0.203

[a] For completeness of information, the activity values given for *A. eutrophus* were taken from reference 7.
[b] Lithoautotophic growth.
[c] Organoautotrophic growth.
[d] LB, LB medium (induction of the *lac* promoter with 0.5 mM IPTG).

TABLE 4. Activities of PGP in cell extracts of *A. eutrophus* grown with various substrates and in transformant strains of *E. coli*

Strain	Growth substrate	Sp act (U/mg of protein)
A. eutrophus H16	H_2-CO_2[a]	0.30
A. eutrophus H16	Pyruvate	<0.01
A. eutrophus HB9	Fructose-formate[b]	<0.01
A. eutrophus HB9(pHG1)	H_2-CO_2	<0.01
E. coli(pAEC4300)	LB[c]	6.58
E. coli(pAEC4301)	LB	0.03
E. coli(pAEP4320)	LB	<0.01
E. coli(pUC18)	LB	<0.01

[a] Lithoautotophic growth.
[b] Heterotrophic growth with fructose; at an optical density at 436 nm of 1.5, 0.2% (wt/vol) formate was added.
[c] LB, LB medium (induction of the *lac* promoter with 0.5 mM IPTG).

```
Ae  MN------APERIDSAARCANALRFLAADAVEQAKSGHPGAPMGMAEMA-EVLWRRHLRHNPANPAWPDR  63
Rs  MK------DIGAAQET-RMANAIRALAMDAVEKAKSGHPGMPMGMADVA-TVLFNRFLTVDPSAPKWPDR  62
Ec  MSSRKEL-----------ANAIRALSMDAVQKAKSGHPGAPMGMADIA-EVLWRDFLKHNPQNPSWADR  57
Sc  MT------QFTDIDKLA--VSTIRILAVDTVSKANSGHPGAPLGRSPAAHVLGESNAHEPNQPKTGSTEI  62
Hp  MSMRIPKAASVNDEQHQRIIKYGRALVLDIVEQYGGGHPGSAMGAMAIG-IALWKYTLKYAPNDPNYFNR  69
Sp  MS------NLS--------VNAIRFLGIDAINKANSGHPGVVMGAAPMAYSSLQNNFISIQLNQTGLTAT  56
                                        ↑
                                      His-30

Ae  DRFVLSNGHASMLQYALLHLTGY-DLPMSQLRQFR--QLHAATPGH-PELGVTP-GVETTTGPLGQGLAN 128
Rs  DRFVLSAGHGSMLLYAIHHLLGYADMDMDQIRSFR--QLGARTAGH-PEYGHAE-GIEVTTGPLGQGIAT 128
Ec  DRFVLSNGHGSMLIYSLLHLTGY-DLPMEELKNFR--QLHSKTPGH-PESGVTPLGVETTTGPLGQGIAN 123
Sc  DLSCLTVTRS--LCCIYLHLTGY-DLSIEDLKQFR--QLGSRTPGH-PEFEL-P-GVEVTTGPLGQGISN 124
Hp  DRFVLSNGHVCLFQYIFQHLYGLKSMTMAQLKSYHSNDFHSLCPGH-PEIEHDA--VEVTTGPLGQGISN 136
Sp  ALFF----QQVMVQCSFMLFFTFLVLKMSAWMRLRV-SVNGVKNTRSPRICHTA-GIDATTGPLGKDF-N 119
                    ↑              ↑             ↑              ↑ ↑
                  His-79         Arg-94        His-103       Gly-116 Leu-118

Ae  AVGMALAEKLLAATFNRP-GFDIVDHHTYVFLGDGCLMEGLSHEACSLAGTLRLGKLIC-LYDDNGISID 196
Rs  AVGMALAERMKNARY----GDDLVDHFTYVIAGDGCLMEGISHEAIDMGGHLGLGRLIV-LWDDNRITID 193
Ec  AVGMAIAEKTLAAQFNRP-GHDIVDHYTYAFMGDGCMMEGISHEVCSLAGTLKLGKLIA-FYDDNGISID 191
Sc  AVGMAMAQANLDMPLTTSRAFTLSDNYTYVFLGDGCLQEGISSEASSLAGHLKLGNLIA-IYDDNKITID 193
Hp  SVGLAIATKNLAATYNKP-GFDIITNKVYCMVGDACLQEGPALESISLAGHMGLDNLIV-LYDNNQVCCD 204
Sp  CYWFCPSRRFLAAKYNRE-GYNIFDHYTYVICGDGDLMEGVSSEAASYAG---LQNLISWLF----FMIQ 181
                                  ↑↑    ↑                      ↑  ↑  ↑  ↑
                               Asp-158  Glu-163                Asp-186  Ile-190
                                  Gly-159                          Asn-188 Ile-192

Ae  GEVAGWFADDT---PKRFAAYG---WHVIADVDGHD-AHALDAALHEAKAERDRPTLICCRTVIGKGAPA 259
Rs  GDSGISTSTDQ---KAPFAASG---WHVLA-CDGHA-PEEIAAAIEAARRDP-RPSMIACRTVIGYGAPN 254
Ec  GHVEGWFTDDT---AMRFEAYG---WHVIRDIDGHD-AASIKRAVEEARAVTDKPSLLMCKTIIGFGSPN 254
Sc  GATSISFDED---VAKRYEAYG---WEVLYVENGNEDLAGIAKAIRQRKLSKDKPTFDQ-NDHNHWLRFL 256
Hp  GSVDIANTED---ISAKFKAC---NWNVIEVENASEDVATIVKALEYAQAEKHRPTLINCRTVIGSGAAF 268
Sp  -MISTWMVRQRIPLQKVFVTVTMLRLHTALVENGTD-LEAIHAAIETAKA-SGKPSLIEVKTVIGYGSPN 248

Ae  KAGGHDVHGAPLGAPEIAAMRTALGWEAE-PFTVPADVADAW-D-ARAQGAAREAEWEARFVSYCAAHPE 326
Rs  KQGGHDVHGAPLGAAEIAAARERLGWDHP-PFEIPADLYEAW-GRIAARGADARAAWETRLQA-----SP 317
Ec  KAGTHDSHGAPLGDAEIALTREQLGWKYA-PFEIPSEIYAQW-D-AKEAGQAKESAWNEKFAAYAKAYPQ 321
Sc  RSGSHSVHGAPLKADDVKQLKSKFGFNPDKSFVVPQEVYDHYQKTILKPGVEANNKWNKLFSEYQKKFPE 326
Hp  ENHCAA-HGNALGEDGVRELKIKYGMNPAQKFYIPQDVYDFFKEKP-AEGDKLVAEWKSLVAKYVKAYPE 336
Sp  KQGTNAVHGAPLGADETASTRQALGWDYE-PFEIPEQVYADFKEHVADRGASAYQAWTKLVADYKEAHPE 317
          ↑
        His-264

Ae  LAEEFVRRANGR---LPEGFDAELMALLDAPSPLQGKIATRKASQLCLEALTPALPELLGGSADLTGSNL 393
Rs  LRAAFETAEAADTSALPPAIAA-YKARLSAEAP---KVATRKASEMALGVVNEALPFAVGGSADLTGSNL 383
Ec  EAAEFTRRMKGE---MPSDFDAKAKEFIAKLQANPAKIASRKASQNAIEAFGPLLPEFLGGSADLAPSNL 388
Sc  LGAELARRLSGQ---LPANWESKLPTY----TAKDSAVATRKLSETVLEDVYNQLPELIGGSADLVLPIL 389
Hp  EGQEFLARMRGE---LPKNWKSFLPQ-----QEFTGDAPTRAAARELVRALGQNCKSVIAGCADLSVSVN 398
Sp  LAAEVEAIIDGRDPV--EVTPADFPALENGFSQ-----ATRNSSQDALNVVAAKLPTFLGGSADLAHSNM 380
                                                    ↑↑
                                                 Asp-383 Leu-384
```

FIG. 4. Alignment of the amino acid sequences (one-letter code) of the chromosomally encoded TK from *A. eutrophus* H16 (Ae), TKs from *R. sphaeroides* (Rs [9]), *E. coli* (Ec [43; GenBank accession number, X68025]), and *S. cerevisiae* (Sc [14]), dihydroxyacetone synthase from *H. polymorpha* (Hp [19]), and the RecP protein from *S. pneumoniae* (Sp [36]). Residues identical to those in TK of *A. eutrophus* are printed in boldface type. Residues in the enzyme from *S. cerevisiae* involved in cofactor or substrate binding and catalysis are indicated below the sequence. Dashes indicate gaps introduced to maximize alignment.

(about 95% nucleotide identity), only the chromosomal gene seems to be functional in both *A. eutrophus* and the foreign host *E. coli*. A possible explanation for this observation is the dramatic difference in the mRNA stabilities of the two genes, although the formation of an enzymatically inactive plasmid-encoded *cbbZ* product in *A. eutrophus* cannot be discounted.

Although PGP has been isolated from many organisms and characterized, only little is known about its structure. The

```
Ae  TNVKASVWVNH----------AG-HGNYVSYGVREFGMAAAMNGIALHG-G-LIPYGGTFMTFSDYSRNA 451
Rs  TRSKGMVSVAP----------GAFAGSYIHYGIREHGMAAAMNGIALHG-G-LRPYGGTFMAFADYCRPS 443
EC  TLWSGSKAINE----------DA-AGNYIHYGVREFGMTAIANGISLHG-G-FLPYTSTFLMFVEYARNA 445
Sc  TPSNLTRWKEALSFQPPSSGSGNYSGRYIRYGIREHAMGAIMNGISAFG-ANYKPYGGTFLNFVSYAAGA 458
Hp  LQWPGVKYFMDPSLSTQCGLSGDYSGRYIEYGIREHAMCAIANGLAAYNKGTFLPITSTFFMFYLYAAPA 469
Sp  TYIKTDGLQDD----------ANRLNRNIQFGVREFAMGTILNGMALHG-G-LRVYGGTFFVFSDYVKAA 439
                                ↑     ↑                        ↑  ↑  ↑
                           Ile-418 Glu-424                Phe-448   Tyr-454
                                                              Phe-451

Ae  IRMAALMRLRVVHVLTHDSIGLGEDGPTHQPVEHAASLRLIPNNQVWRPCDGAETAYAWLAALRREDGPS 521
Rs  IRLSALMGVPVTYVMTHDSIGLGEDGPTHQPVEHLASLRAIPNLAVIRPADAVETAEAWEIAMTATSTPT 513
Ec  VRMAALMKQRQVMVYTHDSIGLGEDGPTHQPVEQVASLRVTPNMSTWRPCDQVESAVAWKYGVERQDGPT 515
Sc  VRLSALSGHPVIWVATHDSIGVGEDGPTHQPIETLAHFRSLPNIQVWRPADGNEVSAAYKNSLESKHTPS 528
Hp  IRMAGLQELKAIHIGTHDSINEGENGPTHQPVESPALFRAYANIYYMRPVDSAEV-FGLFQKAVELPFSS 538
Sp  VRLSALQGLPVTYVFTHDSIAVGEDGPTHEPVEHLAGLRAMPNLNVFRPSRCAWNEAAWYLAVTSEKTPT 509
                           ↑↑   ↑
                      Glu-482   His-487
                           Asp-483

Ae  CLVLSRQALMPFERNPAQRAEIARGGYVLRDVPAPRVV--LIATGSEVEIAMRAALDLADAGIAARVVSM 589
Rs  LLVLSRQNLPTVRTEHRDENLTARGAYLLRDPGERQVT--LIATGSELELALAAADLLAAEGIAAAVVSA 581
Ec  ALILSRQNLAQQERTEEQLANIARGGYVLKDCAGQPELI-FIATGSEVELAVAAYEKLTAEGVKARVVSM 584
Sc  IIALSPDK-TCHNWKVALIESASKGGYVLQDVANPDII--LVATGSEVSLSVEAAKTLAAKNIKARVVSL 595
Hp  ILSLSRNEVLQYLASRAQRRRNA-AGYILEDAENAEVQ--IIGVGAEMEFADKAAKILGRK-FRTRVLSI 604
Sp  ALVLTRQNLTV--EDGTDFDKVAKGAYVVYEMQRPTLIPSLIATGSEVNLAVSAAKELASQGEKSRVVSM 577

Ae  PCVELFYAQDVAYRDTVLPPGLPR---VSVEAGGTWFWRGVVGEQGLALGIDTFGESAPAEALYQHFGLT 656
Rs  PCFELFAAQPADYRATVLGRA-PR---VGCEAALRQGWDLFLGPQDGFVGMTGFGASAPAPALYQHFNIT 647
Ec  SSTDAFDKQDAAYRESVLPKAVTAR--VAVEAGIADYWYKYVGLNGAIVGIETFGESAPAELLFEEFGFT 652
Sc  PDFFTFDKQPLEYRLSVLPDNVPI---MSVEVLATTCWGKYAHQ---SFGIDRFGAPVRHQKSSSSSVSP 659
Hp  PCTRLFDEQSIGYRRSVLRKDGRQVPTVVVDGHVAFGWERYATAS---YCMNTYGKSLPPEVIYEYFGYN 671
Sp  PSTDVFDKQDAAYKEEILPNAVRR--RVAVEMGASQNWYKYVGLDGAVLGIDTFGASAPAPKVLAEYGFT 645

Ae  PAHVAAAARV----------LLEEA---------------   671
Rs  AEAIVKSAK-------------ERI---------------   659   55 % identity to Ae
Ec  VDNVVAKAKE----------LL------------------   664   59 % identity to Ae
Sc  QKVLLKELKR--------PL--HSIRVTS-----------   678   41 % identity to Ae
Hp  PATIAKKVEAYVRACQRDPLLLHDFLDLKEKPNHDKVNKL   711   37 % identity to Ae
Sp  VENLVKIVRN---------LK-------------------   657   40 % identity to Ae
```

FIG. 4—*Continued.*

specificity of PGP (at least of the enzymes from spinach and erythrocytes) is known to be rather limited to the hydrolysis of 2-phosphoglycolate and ethylphosphate. PGP from erythrocytes also hydrolyzes L-phospholactate (37). Studies on the enzymes from the green alga *Chlamydomonas reinhardtii* and the cyanobacterium *Anacystis nidulans* revealed somewhat different properties with regard to stability and pH optimum, but these enzymes generally resemble those of higher plants (18). Compared with the M_r of native PGP from plants and green algae (81,000 to 93,000 [10]), the M_r of the *A. eutrophus* enzyme seems to be significantly lower (70,500). The presumed homotrimeric structure is rather unusual, although other PGP enzymes have not been studied in this respect.

CbbZ sequence alignments with other known gene products revealed only few overall resemblances. However, some similarities to sequence motifs within potential products of a few completely or incompletely sequenced open reading frames were found, with overall identities of up to 30% (Fig. 5). In all cases, either the functions of the encoded proteins are not known yet or the open reading frames were not even identified to code for functional proteins. The *cbbY* gene is located approximately 6 kb upstream of *cbbZ* (27). Its product, CbbY, is still of unknown function but showed a remarkable overall similarity of 27% to PGP without exhibiting any detectable PGP activity. Nevertheless, PGP and CbbY from *A. eutrophus* may have certain structural or functional relationships. Pep27 (30% identity relative to PGP) is encoded within the *E. coli dam* operon (33). Its gene is closely linked to a gene showing a high level of similarity to *cbbE* of *A. eutrophus*, which encodes ribulose-5-phosphate 3-epimerase (27). Similar sequence motifs were found in a potential gene product that is encoded downstream of the folylpoly-γ-glutamate synthetase gene of *Lactobacillus casei* (45). The His3 protein from the plant-pathogenic fungus *Phytophthora parasitica* is an imidazoleglycerolphosphate dehydratase with a rather unusual N-terminal amino acid sequence that is not present in other dehydratases (4). This N terminus shares similarities with CbbZ. Most of the sequence motifs also appear to be conserved in the human GS1

```
Ae  MATVSMPCTAVLIDLDGTLVDSAP----DIVEAANRM--LADFGSPALPFDTVAGFIGRGVPNLVRRVL-  63
Aey MQ-------ALIFDVDGTLADTES----AHLQAFNAAFAEVGLDW---------YWDAPLYTRLLKVAGG  50
Ec  MNKFE-DIRGVAFDLDGTLVDSAP----GLAAAVDMA--LYALELPVAGEERVITWIGNGADVLMERALT  63
Pp                  GTLIDSVP----DLAAAVDRM--LLELGRPPADLEAVRHWVGNGAQVLVRRALA  48
Dm                                 EFI--LEAVVGTGLSREDAARFIE------VRRKLV  28
Lc  MTA------TVIFDLDGTLVNTEA----LYLKSNVKAAAVLGLHRTEADFRPLVGSAGPSEAKIIADLVG  60
Pa  MAS---PVQALLLDMDGVMAEVSQ----SYRQAI--------------IDTARHFGVSVTHEDIDHTKL  48
Hs              MDGLLLDTERLYSVVFQEICNRY--DKKYSWDVKSL--VMGKKALEAAQIIIDVL-  51

Ae  --------------------ETAGLTPRVEAAEAV----AMFHRHYAETNGRLGS-----VF-P-GVEAG 102
Aey KERLMHYWRMVDPEEARGC----KVKETIDAVHAIKT------RHYAERVGAGGL-----PLRP-GIARL 104
Ec  -------WARQERATQRKTMGKPPVDDDIPAEEQVRILRKLFDRYYGEVAEEGTF-----LF-P-HVADT 119
Pp  -----------------GGIEHDAVDDVLAEQGLA-----LFMEAYAQ-SHELTV-----VY-P-GVKDT  88
Dm  QEN----WFKHVR-------------------------------------------------LF-P-DVIPV  45
Lc  ADHAA--WFQQ-----------------FSTQDVL-----------DQIRSGADF-----VL-P-GADKT  93
Pa  AGNANNDW-QLTHRLVVDGLNGASSAPTLEA---V---TAQFEALYQGVGNTLGLCELETLLTPKGLLRE 111
Hs  --------------------QLP-MSKEELVESQ----TKLKEVFP-----MAA-----LM-P-GAEKL  84

Ae  LEALRRQGYRLACVTNKPRA-LAVPLLAL---TGLSQYLEVLVAGD--SIAQMKPDPEPLRHACNLLDVD 166
Aey IDEAGEAGLPLAIATTTTPA-NLDALLQAPLGADWRRRFAAIGDAG--TTAIKKPAPDVYLAVLERLGLE 171
Ec  LGALQAKGLPLGLVTNKPTP-FVAPLLEA---LDIAKYFSVVIGGD--DVQNKKPHPDPLLLVAERMGIA 183
Pp  LRWLQKQGVEMALITNKPER-FVAPLLDQ---MKIGRYFRWMIGGD--TLPQKKPDPAALLFVMQMAGVT 152
Ps     LQKMGVEMALITNKPER-FVAPLLDE---MKLGRFFRWIIGGD--TMPQKKPDPAALFFVMKMAGVP  61
Dm  LLQLSSTGYMLGVASSSIRE-RVELFLSH---LGVSGYFKVVSGLE--PGVKGKPEPDVIVNALKAAGVP 109
Lc  LQTLDQMGYRLALATSSAKH-YVDVVLAA---TGWVKRFDPILTGS--DVTAHKPDPE--IYHVMKTKLP 155
Pa  LHRRQPKG--MAVVTGRPRK-DCAKFLTTH---GIEDLFPVQIWLE--DCPP-KPSPEPILLALKALGVE 172
Hs  IIHLRKHGIPFALATSSGSASFDMKTSRH---KEFFSLFSHIVLGDDPEVQHGKPDPDIFLACAKRFSPP 151

Ae  TA--QGVLVGDSAVDVAAARAAGIPVCLVRYGYAGPGGPAALGADALLDSLEAL-PA-------LLTPAR 226
Aey GG--DCLAIEDSANGLRAARAAGIPTVVTPTAFSAQ-----------DSFEGAL---------LVLPHL 218
Ec  PQ--QMLFFGDSRNDIQAAKAAGCPSVGLTYGYNYGEAIDLSQPDVIYQSINDLLPA-------LGLPHS 244
Pp  PQ--QSLFVGDSRSDVLAAKAAGVQCVGLTYGYNHGRPIHDETPSLVIDDLRALLPGCEDPATGITLADL 222
Ps  AS--QALFVGDSRSDVQAAKAAGVACVALSYGYNHGRPIAEENPAMVIDDLRKLIPGCLDMDAEILLPDI 182
Dm  RS--EALYVGDRMVDCIAARRAGVKVVIVDRGSVNRWKQEECVPDAWISSL-------------LELVDD 164
Lc  ET--PAIVVEDTHVGVAAAEGAGLPVVMIP---GIGQGPDHKATAILAAAITDL-PD------------- 206
Pa  AC--HAAMVGDTVDDIIAGRKAGA----VAYGVLTPQTYAKSILEQTPAAIGKVL---EQVGASVVLT-- 231
Hs  PAMEKCLVFEDAPNGVEAALAAGMQAVMVPDGNLSRDLTTK--ATLVLNALQDFQPE------LFGLPSY 213

Ae  LAPAA--------------------------------  231
Aey GDPGEPMPQHVPGAANRW-ADLAALRAWHHGTLIEAT  254
Ec  ENQE-SKND-----------------------------  252
Pp  QASQ-DRESTVAVTGKFWMKVIKALARWRWRA-----  251
Ps  KRPS-PRESIVVVTRKLWMKVIKALARWRWRA-----  160
Dm  ASTP-TREATRHD-------------------------  176
Lc  -----------------WLQNHPTFA-----------  215
Pa  --PG---------------------------------  233
Hs  E------------------------------------  214
```

FIG. 5. Alignment of the amino acid sequences (one-letter code) of the chromosomally encoded PGP from *A. eutrophus* H16 (Ae), CbbY from *A. eutrophus* H16 (Aey [27]), Pep27 from *E. coli* (Ec [33; GenBank accession number, Z19601]), potential products of incompletely sequenced open reading frames encoded upstream of the *trpE* operons of *P. putida* (Pp [13]) and *P. syringae* (Ps [42]), a protein encoded in the upstream region of a 16S rRNA gene from *D. mobilis* (Dm [22]), a protein encoded downstream of the *fgs* gene from *L. casei* (Lc [45]), the imidazoleglycerol-phosphate dehydratase from *P. parasitica* (Pa [4; GenBank accession number, Z11591]), and the human GS1 protein (Hs [53]). Residues identical to those in PGP of *A. eutrophus* are printed in boldface type. Dashes indicate gaps introduced to maximize alignment.

protein (53) as well as in the potential products of incompletely sequenced open reading frames located immediately upstream of the *trpE* operons of *Pseudomonas putida* (13) and *Pseudomonas syringae* (42) and within the upstream region of a 16S rRNA gene of *Desulfurococcus mobilis* (22). Although the regions of similarity between PGP and the gene products mentioned are dispersed throughout the total sequences, all the proteins contain three or four such regions (residues 14 to 21, 117 to 120, 150 to 153, and 222 to 236 [numbering of *A. eutrophus* PGP]). Thus, the proteins perhaps share some functional features despite their probably different basic functions. It was surprising that no distinct similarities of PGP to other known phosphatases were found, indicating that the enzyme from *A. eutrophus* possibly is a representative of a new type of phosphatase.

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