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NOTES

Primary Structure and Phylogeny of the Calvin Cycle Enzymes Transketolase and Fructosebisphosphate Aldolase of *Xanthobacter flavus*

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***Xanthobacter flavus*, a gram-negative facultatively autotrophic bacterium, employs the Calvin cycle for the fixation of carbon dioxide. Cells grown under autotrophic growth conditions possess an Fe^{2+}-dependent fructosebisphosphate (FBP) aldolase (class II) in addition to a class I FBP aldolase. By nucleotide sequencing and heterologous expression in *Escherichia coli*, genes encoding transketolase (EC 2.2.1.1.; CbbT) and class II FBP aldolase (EC 4.1.2.13; CbbA) were identified. A partial open reading frame encoding a protein similar to pentose-5-phosphate 3-epimerase was identified downstream from *cbbA*. A phylogenetic tree of transketolase proteins displays a conventional branching order. However, the class II FBP aldolase protein from *X. flavus* is only distantly related to that of *E. coli*. The autotrophic FBP aldolase proteins from *X. flavus*, *Alcaligenes eutrophus*, and *Rhodobacter sphaeroides* form a tight cluster, with the proteins from gram-positive bacteria as the closest relatives.**

Xanthobacter flavus grows autotrophically by using the Calvin cycle for the fixation of CO_2. Only 2 of the 11 enzymes of the Calvin cycle are characteristic for this pathway; the others are also present during heterotrophic growth. The key enzymes of the Calvin cycle, phosphoribulokinase and ribulosebisphosphate carboxylase, are encoded within the *cbb* operon, which is transcribed only during autotrophic growth. Two additional genes are located within this operon: *cbbX*, encoding a protein with unknown function, and *cbbF*, encoding fructosebisphosphatase (27, 29). The transcription of the *cbb* operon is positively regulated by CbbR, a LysR-type transcriptional regulator, which binds to two sites in the *cbb* promoter (47).

During autotrophic growth, *X. flavus* uses two fructosebisphosphatase enzymes with distinct properties. The inducible enzyme encoded by *cbbF* has a high level of sedoheptulosebisphosphatase activity and is stimulated by ATP. The second constitutive fructosebisphosphatase has a low level of sedoheptulosebisphosphatase activity and is not stimulated by ATP (48). In contrast to the fructosebisphosphatase isoenzyme pair, only one phoshoglycerate kinase gene, which is not encoded within the *cbb* operon, is employed by *X. flavus*. The *pgk* gene is constitutively expressed, but the expression level is higher during autotrophic growth than during heterotrophic growth (26).

Little is known about the transketolase (EC 2.2.1.1) and fructosebisphosphate aldolase (FBP aldolase; EC 4.1.2.13) enzymes of *X. flavus*. Like fructosebisphosphatase and phosphoglycerate kinase, these enzymes are involved in both heterotrophic metabolism and the fixation of CO_2 via the Calvin cycle. Two unrelated mechanistically distinct types of FBP aldolase enzymes are encountered in bacteria, archaea, and eukarya (24). Class I FBP aldolases form a covalent Schiff base between the substrate and the α-amino group of a lysine residue during catalysis, whereas the class II enzymes depend on a divalent cation as the electrophile in the catalytic cycle (24).

X. flavus (Table 1) was grown heterotrophically on succinate (10 mM) or gluconate (10 mM) and autotrophically on methanol (0.5% [vol/vol]) at 30°C as described previously (22, 28). The activities of transketolase and FBP aldolase were determined, according to published methods (15, 49), in cell extracts which were prepared by using a French pressure cell as described previously (27). The activity of transketolase was increased sixfold following autotrophic growth on methanol compared with that of heterotrophically grown cells. In sharp contrast, the activity of FBP aldolase (without Fe^{2+}) was the same for both heterotrophic and autotrophic growth. Because the activity of class II FBP aldolase is dependent on Fe^{2+} as the electrophile (24), $FeSO_4$ (700 μM) was included in the reaction assay. Surprisingly, Fe^{2+} did not affect the FBP aldolase activity in the cell extracts of heterotrophically grown cells but stimulated the activity of FBP aldolase in cell extracts of autotrophically grown cells 14-fold (Table 2). These results strongly suggest that *X. flavus* employs a class I FBP aldolase during heterotrophic growth and synthesizes an additional class II FBP aldolase during autotrophic growth. The use of class II FBP aldolase is common among bacterial autotrophs (4, 13, 38, 53). Whether a class I FBP aldolase is used during the heterotrophic growth of these bacteria is unknown. The opposite situation exists in the algae *Chlamydomonas mundana* and *Euglena gracilis*, which use a class II FBP aldolase for

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TABLE 1. Bacteria and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristic(s)	Source or reference
Strains		
E. coli		
DH5α	*supE44* Δ*lacU169* (φ80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Bethesda Research Laboratories
P678-54	F *thr-1 ara-13 leu-6 azi-8 tonA2 lacY1 glnU44 gal6 minB2 rpsL135 malA1 xyl-7 mtl-2 thi-1*	1
X. flavus H4-14	Wild-type strain	23
Plasmids		
pBluescriptKS$^+$	Ap^r *lacZ'*, cloning vector	Stratagene
pCD102	Tc^r *cbbLSXFPTA*	21
pBM1	Ap^r, 4.4-kb *Bam*HI fragment from pCD102 containing *cbbP cbbT cbbA* in the same orientation as *lacZ*	This study
pSB375	Ap^r, 2.7-kb *Eco*RI-*Bam*HI fragment containing *'cbbT cbbA*	This study
pEA11	Ap^r, 1.5-kb *Bss*II-*Xba*I fragment containing *cbbA* in the same orientation as *lacZ*	This study
pET11	Ap^r, 2.1-kb *Sma*I-*Stu*I fragment containing *cbbT* in the same orientation as *lacZ*	This study

heterotrophic metabolism and, like higher plants (20), employ a class I FBP aldolase in the Calvin cycle (9, 32, 34). *Escherichia coli* contains a constitutive class II FBP aldolase, whereas a class I enzyme is induced by gluconeogenic growth conditions (37). At present no clear link between growth conditions and the use of a class I or II FBP aldolase can be established. Biochemical characterization of both classes of FBP aldolases of *X. flavus* will be required in order to establish whether the class II FBP aldolase is better adapted to the autotrophic metabolism of this bacterium.

The *cbb* downstream region encodes two proteins. The 4.4-kb *Bam*HI fragment of pBM1 was analyzed with an *E. coli* minicell-producing strain (Table 1) by a modified procedure of Clark-Curtiss and Curtiss (5) as described previously (8). A unique 42-kDa protein was present in extracts of minicells containing pSB375 and pBM1. Two additional proteins with sizes of 71 and 69 kDa were present in the latter extracts only (Fig. 1B). This shows that a gene encoding a 42-kDa protein is located on the right-hand side of the 4.4-kb *Bam*HI fragment of pBM1. The left-hand side of the *Bam*HI fragment encodes two additional proteins with sizes of 71 and 69 kDa (Fig. 1A). Since this DNA fragment is not large enough to encode two proteins of this size, it is likely that the 71-kDa protein gave rise to the 69-kDa protein via proteolytic degradation, a process which has been observed previously in minicell experiments (8).

Identification of *cbbT*, *cbbA*, and *cbbE* genes. The nucleotide sequence of the *Bam*HI fragment of pBM1 on which the genes encoding the 71- and 42-kDa proteins are located was determined with the automated laser fluorescent DNA sequencer (Pharmacia, Uppsala, Sweden). Dideoxy sequencing reactions were carried out with T7 DNA polymerase, with either 5′-end labelled primers or unlabelled primers and fluorescein-labeled

TABLE 2. Activities of transketolase and FBP aldolase

Enzyme	Fe^{2+} in assay	Enzyme activity (nmol min^{-1} mg of protein^{-1}) on growth substrate		
		Gluconate	Succinate	Methanol
Transketolase	ND[a]	86	88	551
FBP aldolase	−	17	18	25
FBP aldolase	+	18	15	232

[a] ND, not determined.

ATP (51, 54). Two open reading frames (ORFA and ORFB) preceded by plausible ribosome binding sites that were transcribed in the same direction as *cbbP* were identified. ORFA is located 255 bp downstream from *cbbP* and may encode a protein with a molecular mass of 73,236 Da. ORFB may encode a protein with a size of 37,982 Da and is separated from ORFA by 219 bp. The predicted molecular masses of the proteins encoded by ORFA and ORFB are in good agreement with the results from the minicell experiments. It is therefore concluded that ORFA and ORFB represent functional genes.

A search of the PIR database, by using the deduced amino acid sequence of ORFA as the query sequence, revealed extensive similarities with the sequences of other transketolase proteins. The putative transketolase protein of *X. flavus* was most similar to transketolase proteins encoded by the *cbbT* genes of *Rhodobacter sphaeroides* (50% identity) (4) and *Alcaligenes eutrophus* (63% identity) (39); the lowest degree of similarity (29% identity) was observed with the human enzyme (25). A similar database search with the amino acid sequence derived from ORFB as the query sequence resulted in the identification of ORFB as a class II FBP aldolase gene. The similarity of the sequence of FBP aldolase of *X. flavus* to those of other organisms varied between 68% identity (*A. eutrophus*) (38) and 30% identity (*Saccharomyces cerevisiae*) (17). Because *X. flavus* induces the synthesis of class II FBP aldolase only during autotrophic growth, we designated the gene encoding this enzyme as *cbbA* (44). The transketolase-encoding gene identified in this study is located between the known *cbb* genes and *cbbA* (Fig. 1). We therefore designated the transketolase gene as *cbbT* (44).

The 5′ end of an open reading frame which was preceded by a plausible ribosome binding site was detected 267 bp downstream from *cbbA*. The deduced amino acid sequence of this partial open reading frame was 52 and 49% identical to the amino-terminal sequences of autotrophic pentose-5-phosphate 3-epimerase (CbbE) from *A. eutrophus* and *Rhodospirillum rubrum*, respectively (10, 19). The partial open reading frame was therefore tentatively identified as *cbbE* (44).

The plasmids pET11 and pEA11 containing *cbbT* and *cbbA* downstream from the *lac* promoter of pBluescript were transformed into *E. coli* DH5α (Table 1). The addition of isopropyl-β-D-thiogalactopyranoside (IPTG; 1 mM) to *E. coli*(pET11) and *E. coli*(pEA11) during mid-exponentional growth on Luria-Bertani (LB) medium at 37°C (36) resulted in high activi-

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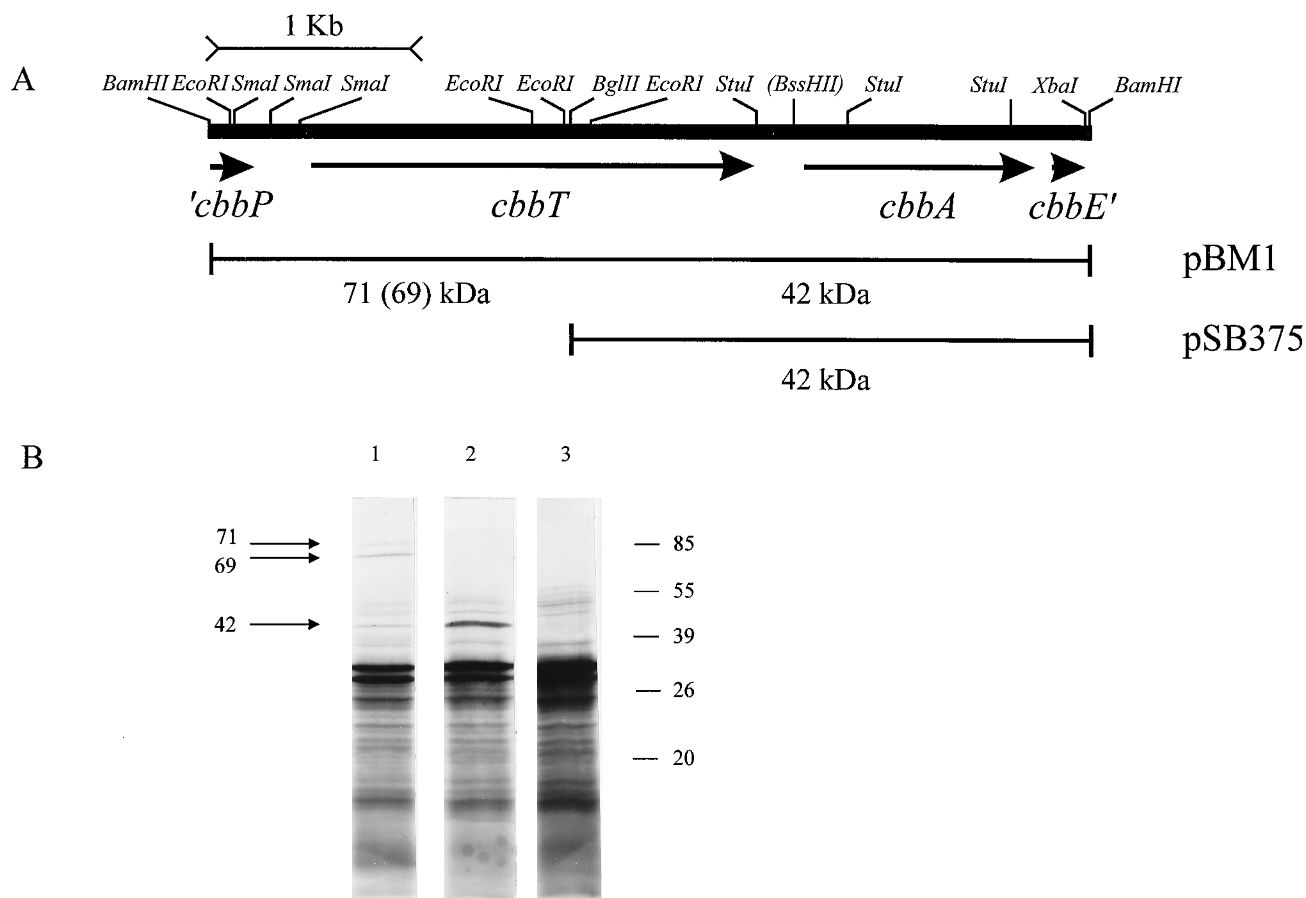

FIG. 1. (A) Restriction map of *Bam*HI fragment containing *cbbT*, *cbbA*, and *cbbE*. The *cbbP* gene was identified previously (27, 29). For the sake of clarity, only one of the *Bss*HII sites which was used in cloning is shown. The molecular masses of the proteins expressed in minicells containing pBM1 and pSB375 are indicated below the bars indicating the size of the DNA insert. (B) Fluorogram of [^{35}S]methionine-labelled proteins from minicells. Lanes: 1, pBM1 (*cbbT cbbA*); 2, pSB375 (*'cbbT cbbA*); 3, pBluescript^{+}. The numbers indicate the sizes (in kilodaltons) the molecular mass markers. Arrows indicate the positions of the proteins encoded by *cbbT* and *cbbA*.

ties of transketolase (395 nmol min^{-1} mg of protein^{-1}) and FBP aldolase (381 nmol min^{-1} mg of protein^{-1}). IPTG-induced *E. coli* (pBluescript) contained threefold less transketolase (114 nmol min^{-1} mg of protein^{-1}) and FBP aldolase (152 nmol min^{-1} mg of protein^{-1}).

The localization of the *cbbT* gene downstream from the conserved *cbbFP* cluster is common (4, 10, 18, 39), with the form I *cbb* operon of *Rhodobacter sphaeroides* and the *cbbFPA* cluster of *Nitrobacter vulgaris* as the only exceptions (13, 43). In contrast, the genetic organization of the *cbbA* (4, 13, 38, 43) and *cbbE* (10, 19) genes with respect to those of the other *cbb* genes is not conserved. The relatively large intergenic regions between the *cbbP*, *cbbT*, *cbbA*, and *cbbE* genes are in sharp contrast to the short distances, varying between 11 and 91 bp, between the previously identified *cbb* genes from *X. flavus*. The transcriptional organization and the potential role of these large intergenic region in the regulation of *cbbT*, *cbbA*, and *cbbE* are currently under investigation.

Phylogeny of FBP aldolase and transketolase proteins. The amino acid sequences of class II FBP aldolase (Fig. 2) and transketolase proteins were aligned by using ClustalW (45). The program Treecon for Windows, version 1.1 (46), was used to analyze the phylogenetic relationships of the FBP aldolase and transketolase proteins. A distance matrix of the FBP aldolase and transketolase protein alignments was calculated by using the model of Dayhoff et al. (6, 14). Insertions and deletions in the sequence alignment were not taken into account. Subsequently, a phylogenetic tree was constructed via the neighbor-joining method developed by Saitou and Nei (35). A bootstrap analysis (1,000 replicates) was carried out to test the reliability of the tree (11). The branching order of the transketolase phylogenetic tree is conventional; the proteins from the proteobacteria, including the enzyme from *X. flavus*, from the gram-positive bacteria, from yeasts, and from mammals each form distinct clusters (data not shown). In contrast, a distance tree of the class II FBP aldolase proteins is unusual in that it does not follow the branching order of a phylogenetic tree based on 16S rRNA alignments (Fig. 3). As has been noted previously (42), the FBP aldolase enzymes from *E. coli* (γ subdivision of proteobacteria) and *Campylobacter jejuni* (ε subdivision of proteobacteria) are more related to those of the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* than to other bacterial enzymes. This has been explained as an example of horizontal gene transfer from bacteria to eukarya (42).

A second anomaly in the FBP aldolase phylogenetic tree is that the FBP aldolase proteins from the proteobacteria are not monophyletic (Fig. 3). Instead, the proteins of *A. eutrophus*, *Rhodobacter sphaeroides*, and *X. flavus*, which belong to the α and β subdivisions of the proteobacteria, have the protein from

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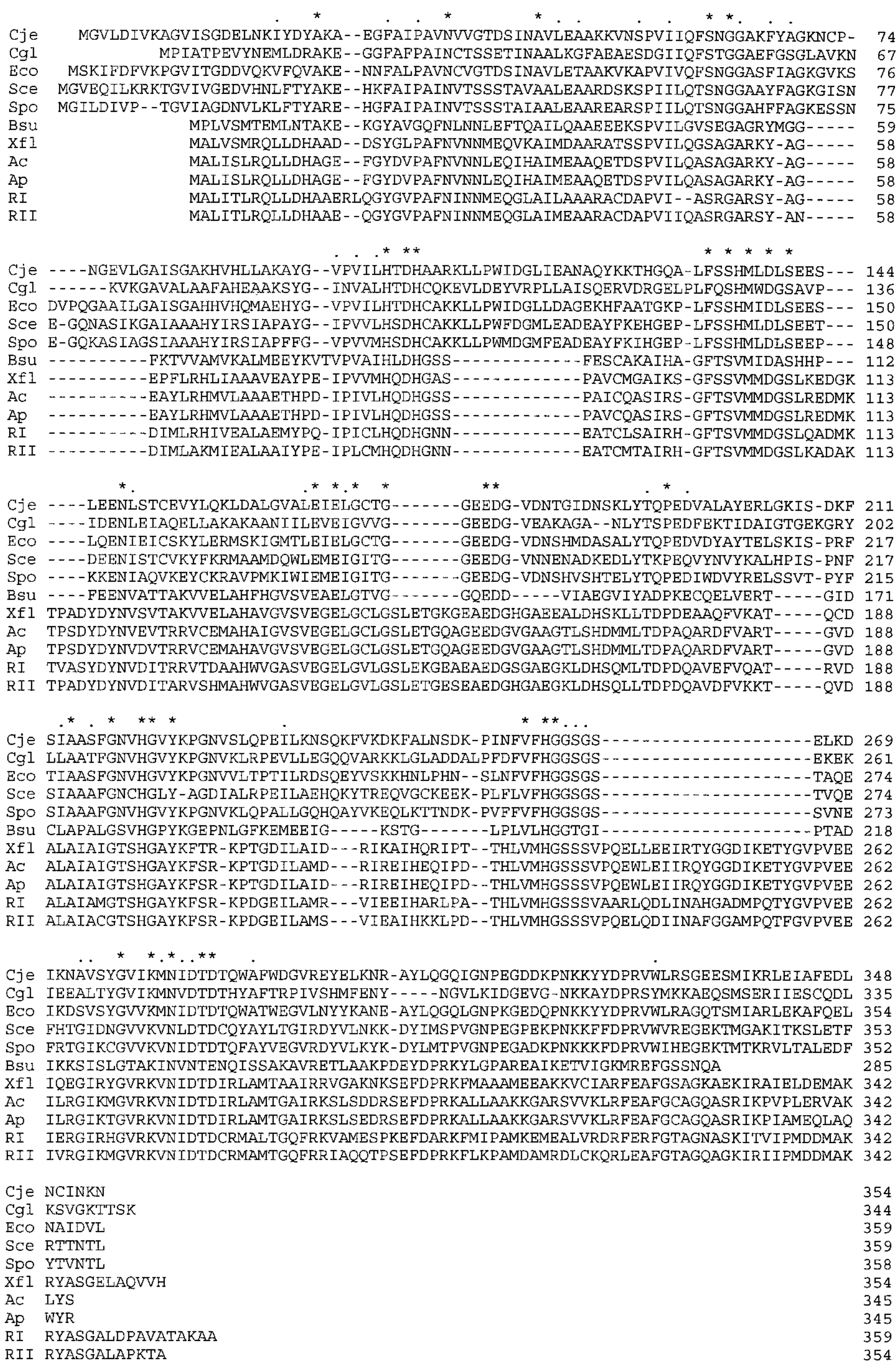

FIG. 2. Alignment of sequence of *X. flavus* FBP aldolase encoded by *cbbA* (Xfl) with those from *A. eutrophus* (Ap [plasmid]; Ac [chromosomal]) (38), *B. subtilis* (Bsu) (30), *Campylobacter jejuni* (Cje) (accession no. X84703), *Corynebacterium glutamicum* (Cgl) (50), *E. coli* (Eco) (2), *Rhodobacter sphaeroides* (RI [form I]; RII [form II]) (4, 13), *Saccharomyces cerevisiae* (Sce) (40), and *Schizosaccharomyces pombe* (Spo) (31). Identical residues are indicated by asterisks; conservative substitutions according to the schemes PAGST, ILVM, QN, ED, HKR, YFW, and C are indicated by dots. The amino acids are represented in one-letter code.

Bacillus subtilis (low G+C subdivision of the gram-positive bacteria) as their closest relative instead of the *E. coli* and *Campylobacter jejuni* enzymes.

The FBP aldolase enzymes from *A. eutrophus*, *Rhodobacter sphaeroides*, and *X. flavus* function only in the Calvin cycle and, unlike the proteins of other organisms, do not play a role in gluconeogenesis or glycolysis. The FBP aldolase sequences of additional proteobacteria remain to be determined in order to ascertain whether the phylogenetic positions of the *E. coli* and *Campylobacter jejuni* enzymes are unusual or, conversely, whether the autotrophic bacteria have acquired a special Calvin cycle FBP aldolase that is related to those from gram-positive bacteria.

The latter scenario is not unlikely, considering the fact that

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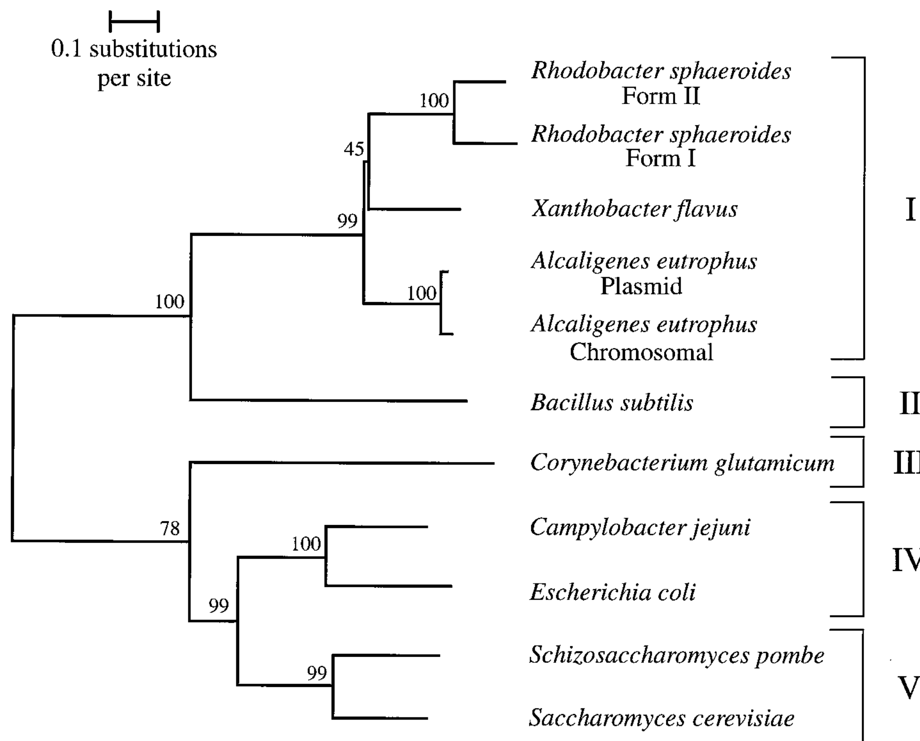

FIG. 3. Unrooted phylogenetic tree based on a distance matrix calculated by using the Dayhoff model (6, 14) and constructed via the neighbor-joining method (35) showing the relationships between class II FBP aldolases. Insertions and deletions in the amino acid alignment were not taken into account. Numbers refer to the percentage of 1,000 bootstrap samples that support each topological element (11). I, α and β subdivision of proteobacteria; II, low G+C subdivision of gram-positive bacteria; III, high G+C subdivision of gram-positive bacteria; IV, γ and ε subdivisions of proteobacteria; V, eukarya.

autotrophic genes are frequently encountered on self-transmissible elements in both gram-positive and gram-negative bacteria. The gram-positive autotrophic bacterium *Nocardia opaca* harbors a selftransmissible genetic element containing *cbb* genes which can be transferred via conjugation to other bacterial species such as *Rhodococcus erythropolis* and *Corynebacterium hydrocarboclastus* (3, 7, 33, 41). Plasmids harboring *cbb* genes which can be transferred via conjugation to other bacteria are frequently encountered in gram-negative bacteria (12, 16, 38, 52). These mobile genetic elements in bacteria provide a means via which lateral transfer of autotrophic genes could have occurred.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper have been entered into GenBank under accession no. U29134.

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