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Cloning, Sequencing, and Overexpression of the Genes Encoding Coenzyme B_{12}-Dependent Glycerol Dehydratase of *Citrobacter freundii*

MARKUS SEYFRIED, ROLF DANIEL, AND GERHARD GOTTSCHALK*

Institut für Mikrobiologie der Georg-August-Universität, 37077 Göttingen, Germany

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The genes encoding coenzyme B_{12}-dependent glycerol dehydratase of *Citrobacter freundii* were cloned and overexpressed in *Escherichia coli*. The B_{12}-free enzyme was purified to homogeneity. It consists of three types of subunits whose N-terminal sequences are in accordance with those deduced from the open reading frames *dhaB*, *dhaC*, and *dhaE*, coding for subunits of 60,433 (α), 21,487 (β), and 16,121 (γ) Da, respectively. The enzyme complex has the composition $\alpha_2\beta_2\gamma_2$. Amino acid alignments with the subunits of the recently sequenced diol dehydratase of *Klebsiella oxytoca* (T. Tobimatsu, T. Hara, M. Sakaguchi, Y. Kishimoto, Y. Wada, M. Isoda, T. Sakai, and T. Toraya, J. Biol. Chem. 270:7142–7148, 1995) revealed identities between 51.8 and 70.9%.

Glycerol dehydratase (EC 4.2.1.30) catalyzes the coenzyme B_{12}-dependent conversion of glycerol, 1,2-propanediol, and 1,2-ethanediol to 3-hydroxypropionaldehyde, propionaldehyde, and acetaldehyde, respectively (14, 23). The enzyme is involved in anaerobic glycerol utilization by *Citrobacter freundii*. In the absence of an external oxidant, glycerol is fermented by a dismutation process using two pathways. Through one pathway glycerol is dehydrogenated by an NAD^+-linked glycerol dehydrogenase to dihydroxyacetone, which is then phosphorylated and funneled to glycolysis by dihydroxyacetone kinase (12). Through the other pathway glycerol is dehydrated by glycerol dehydratase to form 3-hydroxypropionaldehyde, which is reduced to the major fermentation product 1,3-propanediol by the NADH-linked 1,3-propanediol dehydrogenase, thereby regenerating NAD^+ (10). The four key enzymes of this pathway are encoded by the *dha* regulon, the expression of which is induced when dihydroxyacetone or glycerol is present (10, 16). Recently we have cloned and expressed the *dha* regulon of *C. freundii* in *Escherichia coli* (6). The genes coding for 1,3-propanediol dehydrogenase, glycerol dehydrogenase, and dihydroxyacetone kinase have been identified and characterized (5, 7). Here we report on the coenzyme B_{12}-dependent enzyme glycerol dehydratase.

Cloning of the genes encoding glycerol dehydratase. The recombinant cosmid pRD1, which contains a 32-kb insert of *C. freundii* genomic DNA, harbors the entire *dha* regulon of *C. freundii*, as described previously (6). After digestion of pRD1 with *Hin*dIII, ligation into pBluescript SK+, and transformation into *E. coli* DH5α, one subclone with glycerol dehydratase activity (0.5 U/mg) in cell extracts was obtained. Glycerol dehydratase was assayed according to the method of Toraya and Fukui (27). Protein concentrations were determined as described by Bradford (3). The recombinant plasmid recovered from this strain was designated pRD12 and contained a 10.9-kb *Hin*dIII insert of *C. freundii* genomic DNA (Fig. 1). The origin of the cloned DNA was established by Southern blot analysis (data not shown).

Digestion of pRD12 with *Bst*EII and *Xba*I, followed by treatment with Klenow fragment and self-ligation, yielded plasmid pMS2, containing a 5,468-bp *Hin*dIII-*Bst*EII fragment from pRD12. Digestion of pRD12 with *Sma*I, followed by self-ligation, yielded plasmid pMS3, containing a 4,910-bp *Hin*dIII-*Sma*I fragment from pRD12 (Fig. 1). Both recombinant *E. coli* strains exhibited glycerol dehydratase activity (0.5 U/mg).

However, recombinant *E. coli* strains harboring pRD11, which contained a 3,246-bp *Hin*dIII-*Pst*I fragment derived from pRD1 (Fig. 1), showed no glycerol dehydratase activity.

Sequence of the genes encoding glycerol dehydratase. The complete 5,468-bp insert in plasmid pMS2 was sequenced in both directions by the dideoxy chain termination method (18), using [^{35}S]-dATP (DuPont, NEN Research Products, Bad Homburg, Germany) and a Sequenase version 2.0 DNA sequencing kit from U.S. Biochemicals (Braunschweig, Germany) according to the protocol given by the manufacturer, except that the sequencing reaction mixtures contained 10% (vol/vol) dimethyl sulfoxide. As shown in Fig. 1, four successive open reading frames, designated *dhaB*, *dhaC*, *dhaE*, and *orfZ*, were identified.

dhaB codes for a polypeptide of 555 amino acids with a predicted molecular mass of 60,433 Da. This open reading frame is followed by *dhaC* and *dhaE*, encoding polypeptides of 194 and 142 amino acids with calculated molecular masses of 21,487 and 16,121 Da, respectively. *dhaC* and *dhaE* are separated from the upstream open reading frames by 11 and 2 bases, respectively.

dhaE is followed by *orfZ*, whose putative initiation codon was found 10 bases downstream of *dhaE*. *orfZ* encodes a polypeptide of 603 amino acids with a calculated molecular mass of 63,368 Da. Since deletion of 192 bp from the 3′ end of *orfZ* as found in pMS3 (Fig. 1) had no effect on enzyme activity, it was concluded that *orfZ* does not encode a subunit required for glycerol dehydratase activity.

Amino acid sequence homology. The amino acid sequences deduced from *dhaB*, *dhaC*, *dhaE*, and *orfZ* were compared with protein sequences available in the EMBL and GenBank databases. DhaB, DhaC, and DhaE exhibited significant homologies to PddA, PddB, and PddC, respectively, which make up the coenzyme B_{12}-dependent diol dehydratase of *Klebsiella oxytoca* (26) (see Table 1). No significant similarities of either

* Corresponding author. Mailing address: Institut für Mikrobiologie der Georg-August-Universität, Grisebachstrasse 8, 37077 Göttingen, Germany. Phone: 049-551-393781. Fax: 049-551-393793.

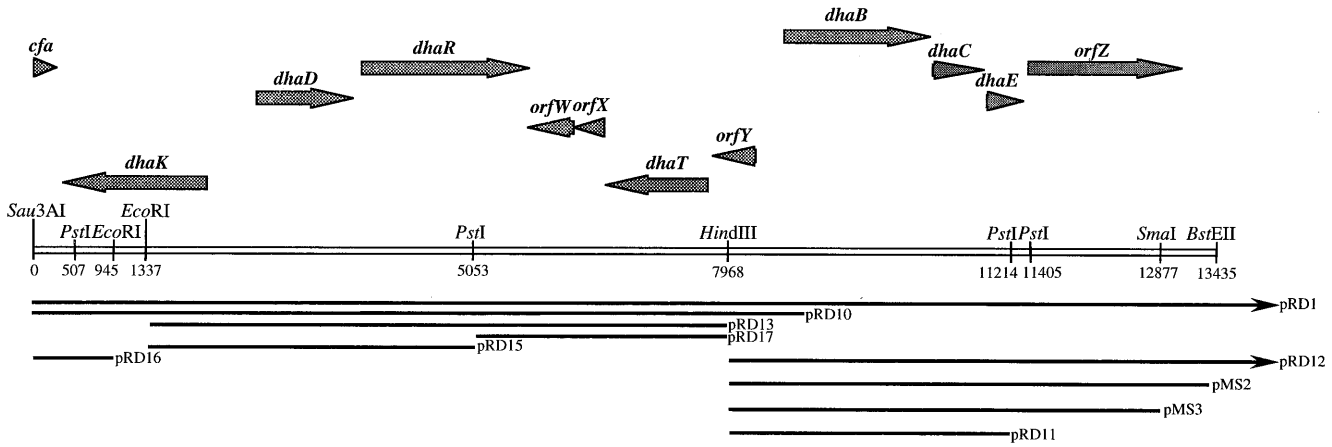

FIG. 1. Apparent gene organization of the *C. freundii dha* regulon. *cfa* encodes cyclopropane fatty acid synthetase; *dhaK* encodes dihydroxyacetone kinase; *dhaD* encodes glycerol dehydrogenase; *dhaR* is a putative transcriptional activator; *dhaT* encodes 1,3-propanediol dehydrogenase; *dhaB*, *dhaC*, and *dhaE* encode subunits of glycerol dehydratase; and *orfW*, *orfX*, *orfY*, and *orfZ* are open reading frames with unknown functions.

dehydratase to other cobalamin-dependent enzymes or cobalamin-binding proteins were found, and no putative binding motif for coenzyme B_{12} matching the conserved sequence described for other cobalamin-dependent proteins was apparent (8, 13). OrfZ revealed no significant homology to any other protein whose sequence is available in the databases.

High-level expression of the genes encoding glycerol dehydratase. To obtain high-level expression of the genes encoding glycerol dehydratase, pMS2 was used to transform *E. coli* K38/pGP1-2, which contains, on plasmid pGP1-2, bacteriophage T7 RNA polymerase under the control of the λp_L promoter and the temperature-sensitive *c*I857 λ repressor (24). Expression of the genes was induced by a shift in temperature from 30 to 42°C as described previously (5). Induction of transformed cells carrying pGP1-2 and pMS2 resulted in inhibition of growth, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed synthesis of three proteins with molecular masses of 60,000, 21,000, and 16,000 Da (Fig. 2). These data are in excellent agreement with those derived from DNA sequencing. Identical results were obtained for *E. coli* strains carrying pMS3 instead of pMS2 (data not shown).

Gel electrophoresis carried out under nondenaturing conditions gave a protein band corresponding to a molecular mass of 190,000 Da, which was not observed when *E. coli* K38/pGP1-2 contained pBluescript SK+ instead of pMS2 (Fig. 3C, lane 1). This band represents glycerol dehydratase, as confirmed by activity staining with 2,4-dinitrophenylhydrazine plus 1,2-propanediol (Fig. 3B). The 2,4-dinitrophenylhydrazone of propionaldehyde was not formed when coenzyme B_{12} was omitted from the reaction mixture (Fig. 3A). The dehydratases of the recombinant *E. coli* strain and of *C. freundii* comigrated during polyacrylamide gel electrophoresis (Fig. 3B, lanes 3 and 4).

A specific glycerol dehydratase activity of approximately 30 U/mg was measured in cell extracts of induced *E. coli*. This is a 15-fold overproduction in comparison with the activity in cell extracts of uninduced *E. coli* and a 30-fold overproduction in comparison with the activity in cell extracts of *C. freundii* grown anaerobically on glycerol.

Enzyme purification. Cells of *E. coli* overproducing glycerol dehydratase were harvested by centrifugation at 6,000 × *g* for 20 min, washed once with 100 mM potassium phosphate buffer (pH 8.0), and resuspended in 1 ml of the same buffer per g (wet weight) of cells. The cells were disrupted by French pressing (1.38×10^8 Pa), and the extract was cleared by centrifugation

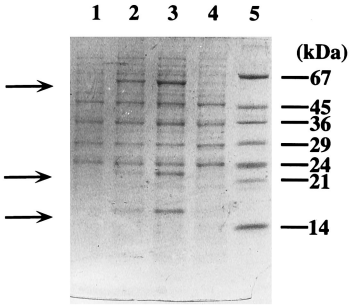

FIG. 2. Overexpression of the genes encoding glycerol dehydratase of *C. freundii*. Extracts of induced *E. coli* were prepared 1, 2, and 3 h after induction (lanes 1, 2, and 3, respectively) and electrophoresed on a 12% polyacrylamide gel under denaturing conditions as described by Schägger and von Jagow (19). Proteins were stained with Coomassie blue (29). Lane 4, extract of uninduced *E. coli*; lane 5, molecular mass markers. Arrows indicate proteins corresponding to subunits of glycerol dehydratase.

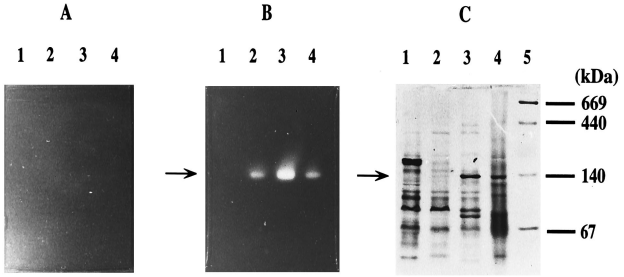

FIG. 3. Activity staining of glycerol dehydratase overproduced in *E. coli*. Extracts of different recombinant *E. coli* strains and of *C. freundii* were electrophoresed under nondenaturing conditions according to the method of Andersson et al. (1). Gels in panels A and B were subjected to activity staining as described by Tobimatsu et al. (26); coenzyme B_{12} was omitted for the gel in panel A. The gel in panel C was stained with Coomassie blue (29). Extracts of *E. coli* K38/pGP1-2/pBluescript SK+ (control, lane 1) and of *E. coli* K38/pGP1-2/pMS2 (lane 3) were prepared 3 h after induction. Lane 2, extract of *E. coli* K38/pGP1-2/pMS2, uninduced culture; lane 4, extract of *C. freundii*, grown anaerobically on glycerol as described previously (5); lane 5, molecular mass markers. Arrows indicate glycerol dehydratase bands.

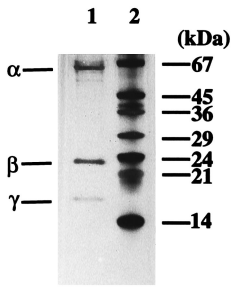

FIG. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified glycerol dehydratase. Proteins were stained with $AgNO_3$ (2). Lane 1, fraction containing purified glycerol dehydratase (after affinity chromatography on vitamin B_{12}-agarose); lane 2, molecular mass markers.

at 32,000 × *g* for 20 min. The extract exhibited a specific activity of 30 U/mg. Two milliliters of this cell extract was treated with ammonium sulfate until 20% (wt/vol) saturation was attained, and then the extract was incubated at 4°C for 30 min. The extract was cleared by centrifugation at 28,000 × *g* and then incubated at 4°C for 20 min. Ammonium sulfate was added to the supernatant (40% [wt/vol] saturation), and the mixture was centrifuged as described above. This partially purified enzyme preparation exhibited a specific activity of 50 U/mg; the recovery rate was 90%. The precipitate was resuspended in 4 ml of equilibration buffer (100 mM glycine-KCl buffer, pH 8.6) and applied to a column packed with 5 ml of vitamin B_{12}-agarose (purchased from Sigma, Deisenhofen, Germany), which had been equilibrated with the same buffer. To obtain purified glycerol dehydratase, two alternative procedures were developed.

(i) Elution with coenzyme B_{12}. After the column was washed with 3 volumes of equilibration buffer, glycerol dehydratase was eluted with 12 ml of equilibration buffer containing 1 mM coenzyme B_{12} (flow rate, 0.5 ml/min). All steps were done under anaerobic conditions in the dark. The final enzyme preparation was homogeneous but enzymatically inactive, and attempts to reactivate it failed. Therefore, an elution procedure avoiding the use of coenzyme B_{12} was developed.

(ii) Elution with NaCl. The column was first washed with equilibration buffer after binding of the protein. The dehydratase was then desorbed with 100 mM glycine buffer (pH 8.6) containing 500 mM NaCl. All steps were done under aerobic conditions in the dark. Active enzyme was recovered by this method. The final enzyme preparation exhibited a specific glycerol dehydratase activity of 226.0 U/mg, with a 32% recovery rate. The dehydratase was contaminated by other proteins to a small extent, but when the column was washed with 100 mM glycine buffer (pH 8.6) containing 500 mM KCl before elution with 500 mM NaCl was carried out, a homogeneous glycerol dehydratase preparation was obtained; the recovery rate, however, was low (1.5%).

The resulting glycerol dehydratase was subjected to polyacrylamide gel electrophoresis under denaturing conditions and yielded three bands with the same molecular masses as were observed during overexpression (Fig. 4). Active and inactive enzyme preparations were indistinguishable with respect to the molecular masses of the three subunits. The N-terminal sequences of the α, β, and γ subunits were identical to those deduced from the sequences of *dhaB*, *dhaC*, and *dhaE*, respectively.

The molecular mass of the holoenzyme was determined by gel permeation chromatography (22) using a fast performance liquid chromatography system with a Superdex 200 HR 10/30 column and a gel filtration calibration kit (Pharmacia, Freiburg, Germany) according to the manufacturer's instructions. MOPS (morpholinepropane sulfonic acid) (50 mM; pH 7.0) containing 100 mM 1,2-propanediol was used as the running buffer. A value of 198,000 Da was calculated for the native glycerol dehydratase.

Determination of the subunit composition. The subunit composition was determined by incorporation of [^{35}S]methionine into glycerol dehydratase during overproduction, using the T7 RNA polymerase-promoter system. *E. coli* strains carrying the genes encoding glycerol dehydratase were grown at 30°C in M9 mineral medium (17) supplemented with 0.05 mM thiamine-HCl, 100 μg each of ampicillin and kanamycin per ml, 0.2% (wt/vol) glucose, 0.3% (wt/vol) glycerol, and a mixture containing all standard amino acids (0.01% [wt/vol]) with the exception of cysteine and methionine. At an A_{600} of 0.5 to 0.7, the temperature was raised to 42°C for 15 min. Rifampin was added (200 μg/ml), and the temperature was maintained at 42°C for 10 min more; then the cultures were shifted to 37°C for 20 min and 100 μCi of [^{35}S]methionine was added. The cultures were incubated at 37°C for 2.5 h, harvested by centrifugation as described above, and resuspended in 200 μl of 100 mM potassium phosphate buffer (pH 8.0). The cells were disrupted by ultrasonication using three 20-s bursts at a power output of 70W with 1 min between bursts.

The ultrasonic extracts were subjected to polyacrylamide gel electrophoresis under nondenaturing conditions. After the gel was stained with Coomassie blue, bands containing the native enzyme were excised, rinsed with denaturing buffer (0.3 M Tris-HCl, 20% [wt/vol] glycerol, 4% [wt/vol] sodium dodecyl sulfate, 10% [vol/vol] 2-mercaptoethanol, and 0.02% [wt/vol] bromophenol blue [pH 6.8]), boiled for 2 min, and loaded onto a sodium dodecyl sulfate-polyacrylamide gel. The subunits were separated electrophoretically, the gel was stained with Coomassie blue, and gel slices (1-mm^2 sections) containing the single subunits were excised. The polyacrylamide gel was solubilized with H_2O_2 as described by Grower and Bransome (11) to quantify the amount of incorporated radioactivity by liquid scintillation counting.

Calculations from the number of methionine residues per subunit derived from DNA sequencing of *dhaB*, *dhaC*, and *dhaE* (25, 5, and 3, respectively) and the radioactivity measured (138,270, 28,030, and 16,260 cpm, respectively) indicate that the subunit stoichiometry of the native enzyme is 1:1:1 (α:β:γ). This suggests a subunit composition of $\alpha_2\beta_2\gamma_2$ (196,082 Da), which is in agreement with the molecular mass of native glycerol dehydratase (198,000 Da) determined by gel permeation chromatography.

Comparison with other coenzyme B_{12}-dependent dehydratases. Glycerol dehydratases have been purified from *Klebsiella pneumoniae* and from *Lactobacillus reuteri* (20, 25). The molecular mass reported for the *K. pneumoniae* enzyme is 188,000 Da (20). It was shown to dissociate into components A and B following ion-exchange chromatography (21), but further studies of the subunit structure have not been carried out. *L. reuteri* glycerol dehydratase was assumed to be a homotetramer of 200,000 Da composed of 52,000-Da subunits (25). Since the genes encoding coenzyme B_{12}-dependent glycerol dehydratase of *C. freundii* have been identified and characterized and the subunit stoichiometry has been determined, more precise figures can now be given for this type of enzyme.

Recently, the genes encoding coenzyme B_{12}-dependent diol dehydratase of *K. oxytoca* were identified (26). Like the *C. freundii* glycerol dehydratase, the *K. oxytoca* diol dehydratase is encoded by three structural genes (*pddA*, *pddB*, and *pddC*), which encode subunits with molecular masses of 60,348 (α),

TABLE 1. Subunit structures of glycerol dehydratase from *C. freundii* and diol dehydratase from *K. oxytoca* and homology between the corresponding gene products

Glycerol dehydratase from *C. freundii* (this study)		Diol dehydratase from *K. oxytoca* (26)		Identity (%)	Similarity (%)
Subunit	Predicted molecular mass (Da)	Subunit	Predicted molecular mass (Da)		
DhaB	60,433	PddA	60,348	70.9	84.8
DhaC	21,487	PddB	24,113	60.5	77.4
DhaE	16,121	PddC	19,173	51.8	70.2

24,113 (β), and 19,173 (γ) Da, respectively (Table 1). A molecular mass of 230,000 Da was determined for the native enzyme (15). The three *K. oxytoca* genes are arranged in the same order as the genes encoding glycerol dehydratase of *C. freundii.*

Sequence homology studies showed that the two dehydratases are very similar. The similarity of the sequence data is in accordance with results obtained from enzyme studies (9, 28). Both enzymes catalyze the coenzyme B_{12}-dependent conversion of 1,2-diols to the corresponding deoxy aldehydes, with glycerol being the preferred substrate for glycerol dehydratase and 1,2-propanediol being the preferred substrate for diol dehydratase, which was also confirmed for *C. freundii* glycerol dehydratase (4). Glycerol dehydratase and diol dehydratase may occur individually or together in various members of the family *Enterobacteriaceae* after induction during anaerobic growth on glycerol. In *K. pneumoniae* ATCC 25955, 1,2-propanediol induces only diol dehydratase, whereas both dehydratases have been found in cells grown on glycerol under anaerobic conditions (9). Glycerol and diol dehydratases of strain ATCC 25955 are distinguishable by their different immunochemical properties (27) and K_m values for coenzyme B_{12} (21 nM and 1.4 μM, respectively) (9).

Nucleotide sequence accession number. The sequence data of the *dha* regulon were submitted to the GenBank database and assigned accession number U09771.

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