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Properties and sequence of the coenzyme B_{12}-dependent glycerol dehydratase of *Clostridium pasteurianum*

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Abstract

The genes encoding coenzyme B_{12}-dependent glycerol dehydratase of *Clostridium pasteurianum* were subcloned and expressed in *Escherichia coli*. The native molecular mass of the enzyme is 190 000 Da. The enzyme converts glycerol, 1,2-propanediol and 1,2-ethanediol to 3-hydroxypropionaldehyde, propionaldehyde and acetaldehyde, respectively, but glycerol is the preferred substrate. The nucleotide sequences of the *dhaBCE* genes encoding the three subunits of glycerol dehydratase and of *orfZ* whose function is unknown were determined. The deduced products of the *dhaBCE* genes with calculated molecular masses of 60 813, 19 549 and 16 722 Da, respectively, revealed high similarity to amino acid sequences of subunits of coenzyme B_{12}-dependent glycerol and diol dehydratases from other organisms.

Keywords: *Clostridium pasteurianum*; Coenzyme B_{12}; 1,3-Propanediol; Glycerol fermentation; Glycerol dehydratase

1. Introduction

Microorganisms such as *Citrobacter freundii, Klebsiella pneumoniae* or *Clostridium pasteurianum* are able to grow anaerobically on glycerol as sole carbon and energy source [1,2]. Glycerol is converted by enteric bacteria to 1,3-propanediol (major product), ethanol, 2,3-butanediol, acetic and lactic acids. The fermentation pattern of clostridia is different in that they form butyric acid as a by-product. Some strains of *C. pasteurianum* produce considerable amounts of butanol and ethanol in addition. Glycerol is fermented by a dismutation process involving two pathways. Through the oxidative branch of the pathway glycerol is dehydrogenated by a NAD^+-linked glycerol dehydrogenase to dihydroxyacetone which is then phosphorylated and funneled to central metabolism by dihydroxyacetone kinase [3]. Through the reductive branch of the pathway, glycerol is dehydrated by the coenzyme B_{12}-dependent glycerol dehydratase to form 3-hydroxypropionaldehyde which is reduced to the fermentation product 1,3-propanediol by the NADH-linked 1,3-propanediol dehydrogenase [4,5]. All four key enzymes and the corresponding genes have been identified and characterized only in 1,3-propanediol-forming enteric bacteria such as *C. freundii* and *K. pneumoniae* [3–7]. In these microorganisms the key enzymes of anaerobic glycerol breakdown are encoded by the *dha* regulon, the expression of which is induced when dihydroxyacetone or glycerol is present.

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In contrast to the 1,3-propanediol-forming enteric bacteria only little information is available about the genes and enzymes responsible for glycerol utilization by clostridia. The activity of all four key enzymes known from 1,3-propanediol-forming enteric bacteria has been determined in crude extracts of *C. pasteurianum* [8]. This was also done for *C. butyricum*, except that the activity of the dihydroxyacetone kinase was not measured [9]. Recently, we have cloned the genes coding for glycerol dehydratase and 1,3-propanediol dehydrogenase of *C. pasteurianum* in *Escherichia coli*. The gene encoding 1,3-propanediol dehydrogenase (*dhaT*) was subcloned and sequenced. The deduced *dhaT* gene product of *C. pasteurianum* exhibited a high level of similarity to the corresponding gene product of 1,3-propanediol-forming enteric bacteria [8]. To our knowledge, no other sequences of genes encoding key enzymes involved in glycerol conversion to 1,3-propanediol by clostridia have been published.

In this report, we describe the sequence of the genes encoding coenzyme B_{12}-dependent glycerol dehydratase of *C. pasteurianum* and the properties of the enzyme produced in recombinant *E. coli*.

2. Materials and methods

2.1. Bacterial strains and vectors

Clostridium pasteurianum DSM 525 and *Citrobacter freundii* DSM 30040 were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). *Escherichia coli* K38/pGP1-2/pMS2 overexpresses the genes encoding glycerol dehydratase of *C. freundii* [4]. *E. coli* ECL707/pFL1 harbors and expresses the genes encoding the reductive branch of glycerol fermentation of *C. pasteurianum* [8]. *E. coli* ECL707 [7], K38/pGP1-2 [10] and JM109 (Promega GmbH, Mannheim, Germany) were used as hosts, and the plasmid pBluescript SK+ (Stratagene GmbH, Heidelberg, Germany) was employed as the vector for cloning and expression experiments.

2.2. Media and growth conditions

C. pasteurianum was grown in a minimal medium according to Kell et al. [11] with 100 mM glycerol as carbon source and *C. freundii* as described previously [6]. *E. coli* was routinely cultivated at 30°C in LB medium [12], which was supplemented with ampicillin (100 μg ml^{-1}) when necessary. Recombinant *E. coli* strains used for expression of the genes involved in glycerol breakdown were grown as described previously [3]. Fermentations were done in anaerobic flasks and media were gassed with N_2 for 30 min before sterilization. In order to prepare cell extracts for enzymatic analysis, cells of the stationary growth phase from 500-ml cultures were harvested by centrifugation at $6000 \times g$ for 20 min, washed twice with 100 mM potassium phosphate buffer, pH 8.0, and resuspended in the same buffer. The cells were disrupted by French pressing (1.38×10^8 Pa), and the extract was cleared by centrifugation at $32\,000 \times g$ for 20 min. All steps were done under anaerobic conditions and at 4°C.

2.3. Molecular procedures

DNA manipulations were done according to standard methods [12]. DNA sequence was determined by the chain termination method of Sanger et al. [13]. The fidelity of the DNA sequence determined for the inserts of pLM1 and pLM2 was confirmed by commercial sequencing (MWG, Ebersberg, Germany). Sequence analysis was performed with the Genetics Computer Group (GCG) program package [14].

As source for subcloning and amplification of the genes encoding glycerol dehydratase the recombinant cosmid pFL1 was used, which harbors the genes encoding the reductive branch of glycerol fermentation of *C. pasteurianum* as described previously [8]. In order to facilitate high level expression of the *dhaBCE* genes by employing the T7 RNA polymerase/promoter system, the plasmid pLM3 was constructed. For this purpose the coding region of *dhaBCE* was amplified from pFL1 by PCR using a set of primers with synthetic restriction recognition sites (underlined): (5′-CCGGGATCCAAGGAGGATTATATATGAAGTCAAA-3′; 5′-CCGGGATCCCTAGTCCTCTATTCTAACTTATTTCTC-3′). The PCR fragment (2722 bp) was digested with *Bam*HI and inserted into linearized pBluescript SK+ in the same orientation as the T7 promoter.

The orientation of *dhaBCE* and DNA sequence fidelity of the insert of pLM3 were confirmed by sequencing.

2.4. Assays

Glycerol dehydratase was assayed according to Toraya et al. [15]. Protein concentrations were determined by the method of Bradford [16] with bovine serum albumin as standard.

2.5. Determination of molecular mass

Electrophoresis under non-denaturing conditions was performed on polyacrylamide gradient slab gels (4–28%) at 4°C in Tris-glycine buffer, pH 8.3 by the method of Andersson et al. [17]. For calculation of the native molecular mass, a commercial high-molecular-mass calibration kit of standard proteins was used (Pharmacia LKB GmbH, Freiburg, Germany). Activity staining of glycerol dehydratase was performed as described by Tobimatsu et al. [18], with 1,2-propanediol as substrate.

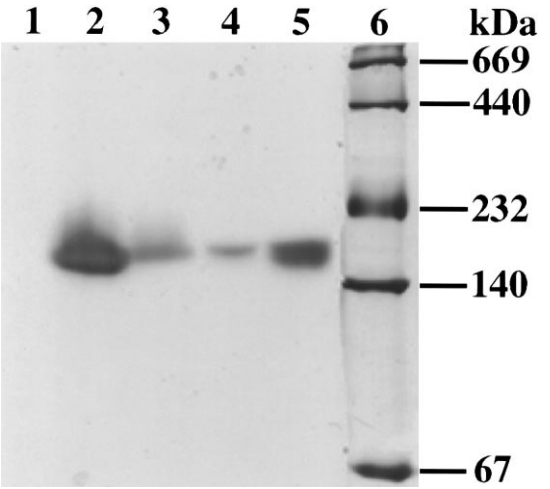

Fig. 1. Non-denaturing polyacrylamide gel electrophoresis and activity staining of glycerol dehydratase. The crude extracts were subjected to electrophoresis under non-denaturating conditions on polyacrylamide gradient slab gels (4–28%). The protein bands were stained as described in Section 2. Lanes: 1, crude extract of *E. coli* ECL707/pWE15; 2, crude extract of *E. coli* K38/pGP1-2/pMS2; 3, purified glycerol dehydratase of *C. freundii*; 4, crude extract of *E. coli* ECL707/pFL1; 5, crude extract of *C. pasteurianum*; 6, molecular mass markers.

3. Results and discussion

The recombinant cosmid pFL1 contains a 13.5-kb insert of *Clostridium pasteurianum* genomic DNA and harbors the genes encoding the reductive branch of glycerol breakdown. The corresponding recombinant *Escherichia coli* strain ECL707/pFL1 exhibited a glycerol dehydratase activity of 1.4 U mg^{-1}. The recorded specific activity was slightly lower than in *C. pasteurianum* [8].

Separation of crude extracts of *E. coli* ECL707/pFL1 by gradient gel polyacrylamide electrophoresis under non-denaturing conditions and activity staining of glycerol dehydratase gave a single band corresponding to a native molecular mass of 190 000 Da (Fig. 1, lane 4). No activity staining was observed when *E. coli* ECL707 containing pWE15 instead of pFL1 was used (Fig. 1, lane 1) or when coenzyme B_{12} was omitted from the reaction mixture (data not shown). The glycerol dehydratases of *E. coli* ECL707/pGP1-2/pMS2, *E. coli* ECL707/pFL1, *C. pasteurianum*, and the purified enzyme of *Citrobacter freundii* comigrated during polyacrylamide gel electrophoresis (Fig. 1, lanes 2–5). Thus, all these enzymes were indistinguishable with respect to their molecular masses. A similar molecular mass (188 000 Da) was also reported for glycerol dehydratase of *Klebsiella pneumoniae* [19]. The mutually related diol dehydratase of *Klebsiella oxytoca* showed a higher native molecular mass of 230 000 Da [20].

The glycerol dehydratase of *C. pasteurianum* and the recombinant enzyme produced in *E. coli* ECL707/pFL1 were capable of catalyzing the coenzyme B_{12}-dependent conversion of glycerol, 1,2-propanediol and 1,2-ethanediol to 3-hydroxypropionaldehyde, propionaldehyde and acetaldehyde, respectively, as is typical of all characterized coenzyme B_{12}-dependent glycerol and diol dehydratases from other organisms. The enzyme of *C. pasteurianum* was most active with glycerol followed by 1,2-propanediol and 1,2-ethanediol. The recorded specific activities were 2.45, 1.58 and 0.48 U mg^{-1}, respectively. Similar results were obtained for the glycerol dehydratase of *C. freundii* [21]. In comparison, diol dehydratases prefer 1,2-propanediol as substrate [22].

To subclone the DNA region encoding glycerol

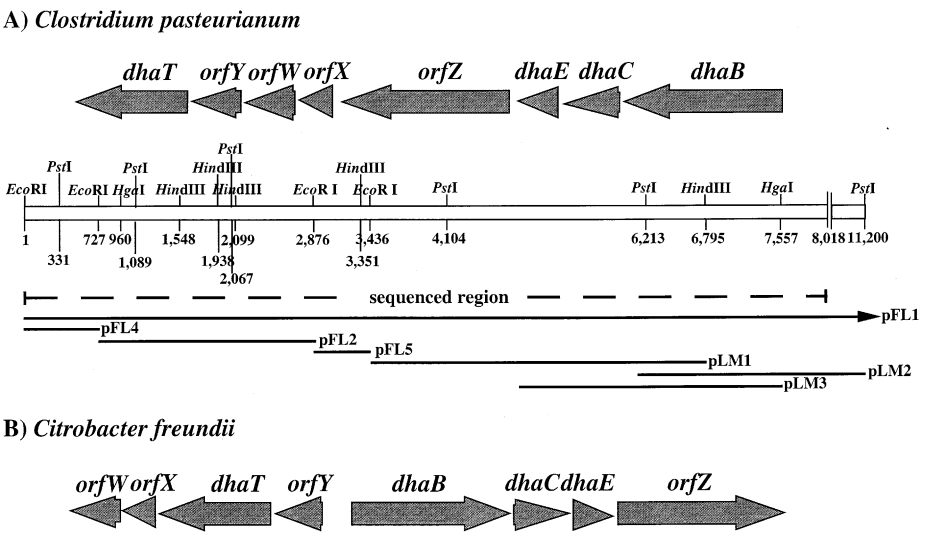

Fig. 2. A: Restriction map and genetic organization of the chromosomal DNA region encoding the reductive branch of glycerol fermentation of *C. pasteurianum*. Arrows and arrowheads represent length, location and orientation of potential genes. The location of genomic *C. pasteurianum* inserts in recombinant plasmids is given below the restriction map. B: Genetic organization of the homologous DNA region from *C. freundii*.

dehydratase of *C. pasteurianum*, pFL1 was digested with *Pst*I, *Eco*RI or simultaneously with *Eco*RI and *Hin*dIII, and ligated into the vector pBluescript SK+, which had been linearized with the corresponding enzymes. Ligated DNA was transformed in *E. coli* JM109. The genes coding for glycerol dehydratase of *C. pasteurianum* were located on 3 recombinant *E. coli* strains with different inserts, one containing a 3359-bp *Eco*RI-*Hin*dIII, and the other two an approximately 5000-bp *Pst*I and a 560-bp *Eco*RI fragment of genomic *C. pasteurianum* DNA. The plasmids isolated from these strains were designated pLM1, pLM2 and pFL5, respectively. The origin and the neighborhood on the chromosome of the cloned fragments was established by Southern blot analysis (data not shown).

The inserts of pLM1 and pFL5 were entirely and the insert of pLM2 was partly sequenced in both directions (Fig. 2A). The restriction map and the apparent gene organization of the entire reductive branch of glycerol fermentation by *C. pasteurianum* are depicted in Fig. 2A. As shown in Fig. 3 four

Table 1
Sequences homologous to the *C. pasteurianum dhaBCE* genes

Organism	Gene name			Gene length (bp)			Protein molecular mass (Da)			Amino acid identity (%)		
	L	M	S	L	M	S	L	M	S	L	M	S
Clostridium pasteurianum	*dhaB*	*dhaC*	*dhaE*	1665	540	441	60 813	19 549	16 722	100	100	100
Citrobacter freundii	*dhaB*	*dhaC*	*dhaE*	1668	585	429	60 433	21 487	16 121	78.1	68.9	65.1
Klebsiella pneumoniae	*gldA*	*gldB*	*gldC*	1668	585	426	60 621	21 310	16 094	78.1	68.9	66.7
Klebsiella oxytoca	*pddA*	*pddB*	*pddC*	1662	672	519	60 348	24 113	19 173	76.3	64.5	58.5
Salmonella typhimurium	*pduC*	*pduD*	*pduE*	1662	672	519	60 307	24 157	19 131	75.9	64.6	58.6

The *dhaBCE* genes of *C. pasteurianum* (this study) and *C. freundii* [4] and the *gldABC* genes of *K. pneumoniae* [23] encode coenzyme B_{12}-dependent glycerol dehydratases. The *pddABC* genes of *K. oxytoca* [18] and the *pduCDE* genes of *S. typhimurium* [25] encode coenzyme B_{12}-dependent diol dehydratases. The values for amino acid identity are given with respect to the *C. pasteurianum* sequence. L, large subunit; M, medium subunit; S, small subunit.

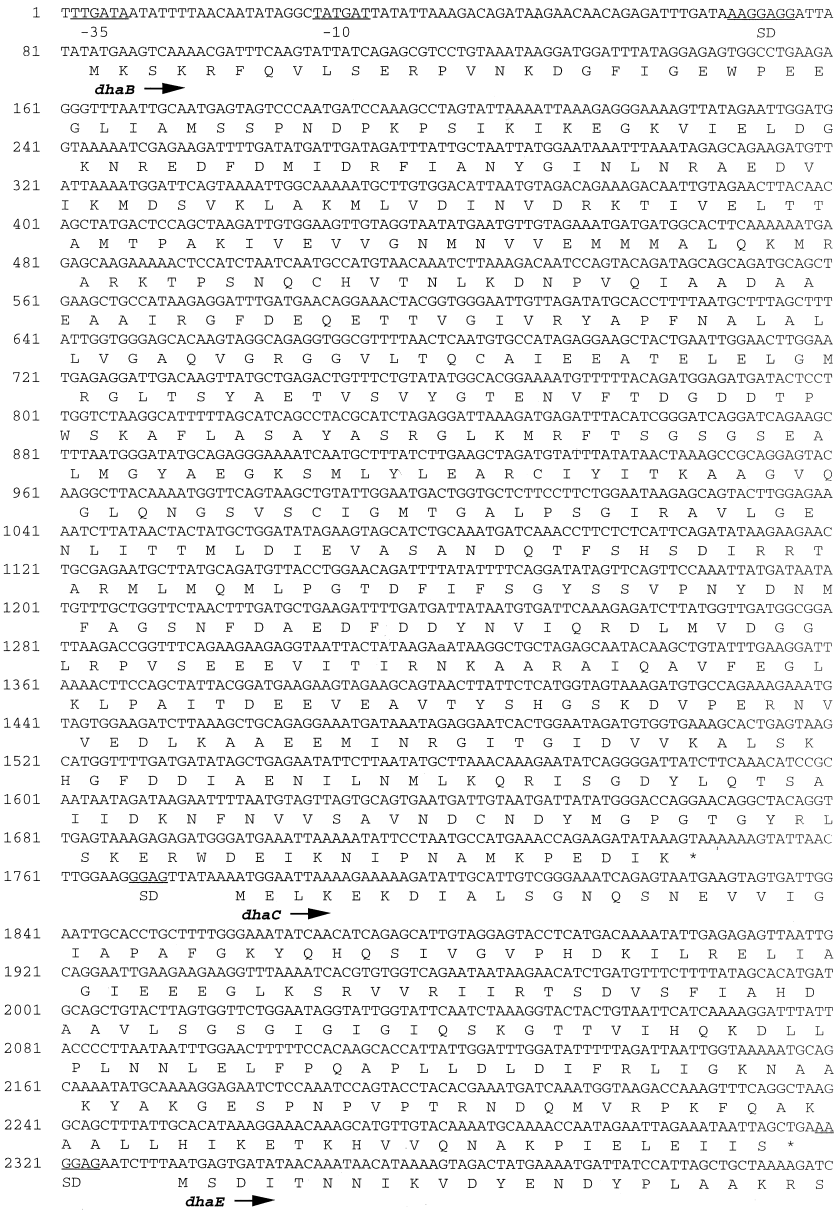

Fig. 3. Nucleotide sequence of the cloned region. Only one strand is shown. The three structural genes for glycerol dehydratase (*dhaB, dhaC, dhaE*) and *orfZ* have been translated by using the one-letter amino acid code; amino acid symbols are written below the first nucleotide of the corresponding codon. Potential ribosome binding sites (SD) and putative σ^{70}-dependent promoters are underlined. The sequence has been submitted to GenBank under accession number AF051373. For positions 2401–4640, see overleaf.

successive potential genes, designated according to the dehydratase genes of *C. freundii* as *dhaB*, *dhaC*, *dhaE* and *orfZ*, were identified within the sequence. All presumptive genes were preceded by a potential ribosome-binding site, appropriately spaced from the start codon (Fig. 3). A conserved sequence for σ^{70}-dependent promoters is located upstream of the *dhaB* gene in positions 2–32 (Fig. 3). The *dhaB* gene (1665 bp) of *C. pasteurianum* codes for a polypeptide of 554 amino acids with a calculated molec-

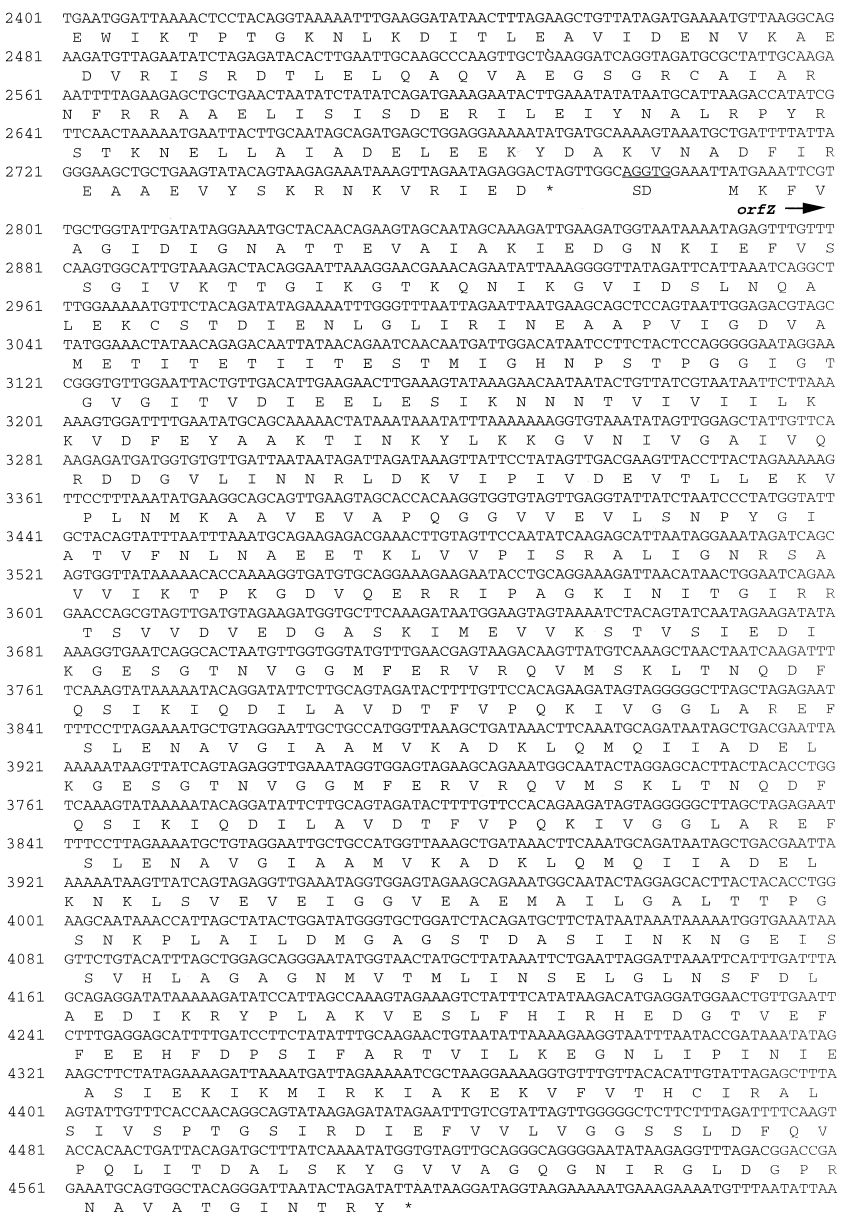

Fig. 3. (*continued*)

ular mass of 60 813 Da. This open reading frame is followed by *dhaC* (540 bp) and *dhaE* (441 bp) encoding polypeptides of 179 and 146 amino acids with predicted molecular masses of 19 549 and 16 722 Da, respectively. *DhaC* and *dhaE* are separated from the upstream open reading frames by 29 and 14 bases, respectively. The *dhaE* gene is followed by *orfZ*, whose putative initiation codon was found 16 bases downstream of *dhaE*. The *orfZ* gene encodes a polypeptide of 602 amino acids with a calculated molecular mass of 64 513 Da.

In order to show that the *dhaBCE* genes of *C. pasteurianum* encode the three structural subunits of glycerol dehydratase, the *dhaBCE* coding region

was amplified by PCR and cloned into pBluescript SK+ in the same orientation as the T7 promoter. The resulting recombinant plasmid designated pLM3 was used to transform *E. coli* K38/pGP1-2, which contains on the plasmid pGP1-2 bacteriophage T7 RNA polymerase under control of the λp_L promoter and the temperature-sensitive *c*I857 λ repressor. Expression of the genes was induced by a shift in temperature from 30 to 42°C [10]. Induction of transformed cells carrying pGP1-2 and pLM3 resulted in retarded growth. Gel electrophoresis carried out under non-denaturing conditions and activity staining revealed that the enzyme produced in *E. coli* K38/pGP1-2/pLM3 has the same native molecular mass as the dehydratase of *C. pasteurianum* (data not shown). A specific glycerol dehydratase activity of 1.9 U mg^{-1} was measured in cell extracts of induced *E. coli* K38/pGP1-2/pLM3. This is a slight (1.4-fold) increase in comparison to the activity in cell extracts of *E. coli* ECL707/pFL1. Since omission of *orfZ* as found in pLM3 (Fig. 2A) did not result in loss of enzyme activity, it was concluded that *orfZ* does not encode a subunit required for glycerol dehydratase activity.

The deduced amino acid sequences of the *dhaBCE* coding regions of *C. pasteurianum* were compared to protein sequences in the EMBL and GenBank databases (Table 1). The deduced amino acid sequence of the *dhaBCE* genes from *C. pasteurianum* are 70.8% identical to DhaBCE from *C. freundii*, 71.2% identical to GldABC from *K. pneumoniae*, 66.5% identical to PddABC from *K. oxytoca*, and 66.4% identical to PduCDE from *Salmonella typhimurium*. The *dhaBCE* and *gldABC* genes encode the three subunits of coenzyme B_{12}-dependent glycerol dehydratases. The *pddABC* and the *pduCDE* genes code for the subunits of coenzyme B_{12}-dependent diol dehydratases. The *dhaBCE* genes of *C. pasteurianum* are arranged in the same order as the three structural genes encoding dehydratases of the other mentioned organisms. In addition, the calculated molecular masses of the corresponding gene products are very similar (Table 1). The sequence comparison of the *orfZ* gene product of *C. pasteurianum* revealed 63.4, 63.0 and 59.2% identity (72.9, 72.7 and 70.2% similarity) to the corresponding homologous gene products of *C. freundii*, *K. pneumoniae* and *K. oxytoca*, respectively. In all these organisms *orfZ* or the homologous gene product is located immediately downstream of the three structural genes for dehydratase. It has been shown for the dehydratases of *C. pasteurianum*, *C. freundii*, *K. pneumoniae* and *K. oxytoca* that this gene product is not required for dehydratase activity [4,18,23]. However, the gene product of *K. oxytoca* is suspected to be involved in reactivation of glycerol-inactivated diol dehydratase or the enzyme-cyanocobalamin complex [24]. The proteins encoded by the *dhaBCE* and *orfZ* were also searched for motifs related to those in the Prosite dictionary by using GCG software. No matches were found.

Since the genes encoding glycerol dehydratase and 1,3-propanediol dehydrogenase of *C. pasteurianum* have been identified and characterized and the properties of the corresponding enzymes have been determined, a comparison of the reductive branch of glycerol utilization by *C. pasteurianum* to 1,3-propanediol-forming enteric bacteria such as *C. freundii* and *K. pneumoniae* can now be done. The deduced amino acid sequences and properties of the gene products are very similar, but the genetic organization is different. In contrast to *C. freundii* (Fig. 2B) and *K. pneumoniae* (not shown) the genes encoding glycerol dehydratase and 1,3-propanediol dehydrogenase of *C. pasteurianum* (Fig. 2A) showed the same orientation and the presumptive genes *orfX*, *orfW*, *orfY* were all located upstream of the *dhaT* gene.

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