Molecular Characterization of the Glycerol-Oxidative Pathway of *Clostridium butyricum* VPI 1718

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The glycerol oxidative pathway of *Clostridium butyricum* VPI 1718 plays an important role in glycerol dissimilation. We isolated, sequenced, and characterized the region coding for the glycerol oxidation pathway. Five open reading frames (ORFs) were identified: *dhaR*, encoding a putative transcriptional regulator; *dhaD* (1,142 bp), encoding a glycerol dehydrogenase; and *dhaK* (995 bp), *dhaL* (626 bp), and *dhaM* (386 bp), encoding a phosphoenolpyruvate (PEP)-dependent dihydroxyacetone (DHA) kinase enzyme complex. Northern blot analysis demonstrated that the last four genes are transcribed as a 3.2-kb polycistronic operon only in glycerol-metabolizing cultures, indicating that the expression of this operon is regulated at the transcriptional level. The transcriptional start site of the operon was determined by primer extension, and the promoter region was deduced. The glycerol dehydrogenase activity of *DhaD* and the PEP-dependent DHA kinase activity of *DhaKLM* were demonstrated by heterologous expression in different *Escherichia coli* mutants. Based on our complementation experiments, we proposed that the HPr phosphoryl carrier protein and His9 residue of the *DhaM* subunit are involved in the phosphoryl transfer to dihydroxyacetone-phosphate. *DhaR*, a potential regulator of this operon, was found to contain conserved transmitter and receiver domains that are characteristic of two-component systems present in the AraC family. To the best of our knowledge, this is the first molecular characterization of a glycerol oxidation pathway in a Gram-positive bacterium.

Glycerol can be utilized as a carbon source by bacteria via several metabolic pathways that convert glycerol to dihydroxyacetone-phosphate (DHAP) before DHAP enters the glycolytic pathway. Under aerobic conditions, *Escherichia coli* phosphorolates glycerol using an ATP-dependent glycerol kinase (19), and glycerol-3-phosphate then is oxidized to DHAP by a membrane-bound FAD-dependent glycerol-3-phosphate dehydrogenase (35). Under anaerobic conditions, *Klebsiella pneumoniae* and *Citrobacter freundii* oxidize glycerol using a soluble NAD"-dependent glycerol dehydrogenase (9, 13), and dihydroxyacetone (DHA) then is phosphorylated by a DHA kinase to DHAP (9, 13, 21). *DHA* kinases can be grouped into two structurally related families according to the source of the high-energy phosphate: ATP or phosphoenolpyruvate (PEP). ATP-dependent DHA kinases are single-polypeptide two-domain proteins (37), while the PEP-dependent DHA kinases consist of three subunits: *DhaK, DhaL*, and *DhaM* (18). *DhaK* and *DhaL* are homologous to the amino-terminal K domain and the carboxy-terminal L domain of the ATP-dependent kinases. *DhaK* contains a binding site for DHA, and *DhaL* contains an ADP binding site. *DhaM* is a phosphohistidine protein that transfers phosphoryl groups from a phosphohistidine carrier protein of the phosphotransferase system (PTS) (HPr or enzyme I) to the *DhaL*-ADP complex (3, 18).

In *E. coli*, genetic and biochemical studies have demonstrated that the *dha* operon is controlled by *DhaR* and the two kinase subunits, *DhaK* and *DhaL* (4, 5). *DhaK* and *DhaL* act antagonistically; *DhaK* functions as a corepressor and *DhaL* as a coactivator of *DhaR* (4). In the presence of DHA, when the phosphoryl group is transferred from *DhaL*::ATP to DHA, the now-dephosphorylated *DhaL*::ADP binds to the *DhaR* receiver domain and activates the expression of the *dha* operon. In the absence of DHA, *DhaL*::ADP is rephosphorylated by *DhaM* to *DhaL*::ATP, which does not bind to *DhaR* (4).

*Clostridium butyricum* VPI 3266 can convert glycerol reductively to 1,3-propanediol and oxidatively via DHAP to acetate and butyrate (14, 31). The physiology of cells metabolizing glycerol has been studied in chemostat cultures (30). Glycerol consumption is associated with the induction of (i) a glycerol dehydrogenase and a dihydroxyacetone kinase that feed glycerol into the central metabolism (30), and (ii) a B12-independent glycerol dehydratase and an NAD"-dependent 1,3-propanediol dehydrogenase involved in propanediol formation (27). Although the molecular characterization of the 1,3-propanol production pathway has provided insight into anaerobic glycerol metabolism in *C. butyricum*, both the identity and the regulation of the genes involved in glycerol oxidation remain to be elucidated.

In this paper, we report the cloning, sequencing, and molecular characterization of the genes encoding glycerol dehydrogenase and *DHA* kinase in *C. butyricum* VPI 1718. Furthermore, we demonstrate that the *C. butyricum* DHA kinase is PEP dependent and obtains its phosphoryl group from the HPr phosphoryl carrier protein. To the best of our knowledge, this
TABLE 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic(s)*</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Clostridium butyricum</em> VPI 1718</td>
<td>Obtained from Virginia Polytechnic Institute in 1990; identical to VPI 3266, except that it is missing pCB101 and pCB102</td>
<td>30</td>
</tr>
<tr>
<td><em>Escherichia coli</em> DH5α</td>
<td>supE44lacU169 (Δ880 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td><em>Escherichia coli</em> BW25113 ΔgldA::km</td>
<td>Deletion of the gldA gene that is replaced by a Km’ marker</td>
<td>2</td>
</tr>
<tr>
<td><em>Escherichia coli</em> BW25113 ΔglpK::km</td>
<td>Deletion of the glpK gene that is replaced by a Km’ marker</td>
<td>2</td>
</tr>
<tr>
<td><em>Escherichia coli</em> BW25113 ΔptsH::km</td>
<td>Deletion of the ptsH gene that is replaced by a Km’ marker</td>
<td>2</td>
</tr>
<tr>
<td><em>Escherichia coli</em> BW25113 ΔgldA ΔglpK</td>
<td>Deletion of the gldA and glpK genes</td>
<td>This study</td>
</tr>
<tr>
<td><em>Escherichia coli</em> BW25113 ΔgldA ΔglpK ΔdhaKLM</td>
<td>Deletion of the gldA, glpK, dhaKLM genes</td>
<td>This study</td>
</tr>
<tr>
<td><em>Escherichia coli</em> BW25113 ΔgldA ΔglpK ΔdhaKLM ΔptsH::km</td>
<td>Deletion of the gldA, glpK, dhaKLM and ptsH genes; ptsH is replaced by a Km’ marker</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pUC18</td>
<td>Amp’, origin ColE1; 2.7 kb; used for construction of the genomic libraries</td>
<td>42</td>
</tr>
<tr>
<td>pGEM-T</td>
<td>Amp’, origin ColE1; 3 kb; used for PCR product cloning</td>
<td>Promega</td>
</tr>
<tr>
<td>pSI-2</td>
<td>pGEMT + 550-bp insert of the PCR-amplified <em>C. butyricum</em> glycerol dehydrogenase gene</td>
<td>This study</td>
</tr>
<tr>
<td>pPSH1</td>
<td>pUC18 + HindIII fragment (2 kbp) containing the <em>C. butyricum</em> glycerol dehydrogenase gene</td>
<td>This study</td>
</tr>
<tr>
<td>pPSB10</td>
<td>pUC18 + BglII fragment (10 kbp) of <em>C. butyricum</em> containing the dhaDKLM operon</td>
<td>This study</td>
</tr>
<tr>
<td>pSE13</td>
<td>pUC18 + EcoRI fragment (7.5 kbp) of <em>C. butyricum</em> containing the upstream sequence of the dhaDKLM operon</td>
<td>This study</td>
</tr>
<tr>
<td>pSOS95 pSOS95del</td>
<td>Ap’, MLS (Em’) acetone operon, repL gene, ColE1 origin pUC18 + replica, and MLS’ marker</td>
<td>12, 39</td>
</tr>
<tr>
<td>pVOK K1-3</td>
<td>pUC18 + replica and gene MLSR’ + <em>C. butyricum</em> synthetic dhaDKLM operon without the dhaD promoter</td>
<td>This study</td>
</tr>
<tr>
<td>pVOK K1-3 ΔD1</td>
<td>pUC18 + replica and gene MLSR’ + <em>C. butyricum</em> synthetic dhaDKLM operon without the dhaD promoter</td>
<td>This study</td>
</tr>
<tr>
<td>pVOK K1-3 ΔD1 H10A</td>
<td>Same as pVOK1-3 ΔD1, but with a mutation of dhaM that replaces the histidine 9 residue of DhaM with an alanine residue</td>
<td>This study</td>
</tr>
</tbody>
</table>

*a* MLS, macrolides-lincosamides-streptogramin B.

is the first molecular characterization of genes involved in a glycerol oxidation pathway in a Gram-positive bacterium.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** All bacterial strains and plasmids used or derived from this study are listed in Table 1. *E. coli* BW25113 ΔgldA ΔglpK, derived from the Keio collection, was constructed by (i) combination of mutations using P1 transduction (32) and (ii) removal of the Km’ marker by FLP recombinase (10). The *E. coli* BW25113 ΔgldA ΔglpK ΔdhaKLM strain was constructed using the gene deletion method previously described (10): (i) replacing the dhaKLM genes with a Km’ marker in the *E. coli* BW25113 ΔgldA ΔglpK strain and (ii) removing the Km’ marker by using FLP recombinase. The *E. coli* BW25113 ΔgldA ΔglpK ΔdhaKLM ΔptsH::km strain was constructed using the P1 transduction of *E. coli* BW25113 ΔgldA ΔglpK ΔdhaKLM with a P1 lysate of the *E. coli* BW25113 ΔptsH::km strain from the Keio collection.

The cloning of the dhaDKLM genes was performed by the PCR amplification of genomic DNA using the Expand Long Template PCR system. A pair of primers that introduced the BamHI and SfoI restriction sites upstream and downstream of dhaDKLM (GDH1 and GDH2) (Table 2) was used. The amplified fragment of 3.2 kb was subcloned into the pGEM-T Easy vector (Promega, Charbonnières, France) and sequenced to ensure that no mutations were introduced. The 3.2-kb BamHI/SfoI fragment cut from the vector was ligated to the 5-kb BamHI/SfoI fragment of pSOS95, yielding the 8.2-kb plasmid pVOK1-3.

For enzyme assays and heterologous gene expression, recombinant *E. coli* BW25113 ΔgldA ΔglpK ΔdhaKLM(pVOK1-3), *E. coli* BW25113 ΔgldA ΔglpK ΔdhaKLM(pVOK1-3 ΔD1), and *E. coli* BW25113 ΔgldA ΔglpK ΔdhaKLM(pVOK1-3 ΔD1) were used.
ΔdhaKL (pSS93del) were grown anaerobically in medium containing (per liter): tryptone, 10 g; yeast extract, 5 g; HEPES, 2.3 g; FeSO₄, 50 mg; nitroliotropic acid (NTA), 200 mg; K₂HPO₄, 0.5 g; NaCl, 2 g; DHA, 2 g; and NaNO₃, 0.85 g. The medium also contained ampicillin (100 μg/mL). The pH of the medium was adjusted to 7.3 by the addition of 6 M NH₄OH.

For the growth complementation of different E. coli mutants, M9 DHA (10 mM) and/or M9 glycerol (10 mM) liquid medium supplemented with ampicillin (100 μg/mL) and/or M9 glycerol (10 mM) liquid medium supplemented with ampicillin (100 μg/mL) was used (32).

### Nuclear acid isolation and manipulation
Chromosomal DNA of C. butyricum VPI 1718 was extracted as described previously (7). The isolation of plasmids from E. coli DH5α was performed using Qiaprep spin mini- and midiprep columns (Qiagen, Courtaboeuf, France). Restriction and modification enzymes were purchased from New England BioLabs (Ozyme; Saint Quentin, France) or Gibco BRL (Eragny, France) and used according to the recommendations of the manufacturer. DNA fragments were extracted from agarose gels using the Qiaquick system (Qiagen). Total RNA of C. butyricum VPI 1718 was extracted from continuous cultures using the RNAeasy midi kit (Qiagen).

### Hybridization
Chromosomal DNA of C. butyricum VPI 1718 was digested to completion with restriction enzymes corresponding to suitable cloning sites in pUC18, and the resulting fragments were separated by agarose gel electrophoresis in 0.5× TAE buffer (20 mM Tris, 20 mM acetate, 0.5 mM EDTA). Southern blotting (32) was performed by capillary transfer to Hybond-N* membranes (Amersham Pharmacia Biotech, Les Ulis, France) and fixation of the separated DNA fragments. To create a DNA probe for Southern blot hybridization, a 1.5-kb internal fragment of the C. butyricum VPI 1718 dhaD and dhaK genes was obtained after HindIII digestion of pSPB10. The probe was radiolabeled with [α-32P]dATP (specific activity, 3,000 Ci/mmol; Amersham Pharmacia Biotech) using the Megaprime DNA labeling system (Amersham Pharmacia Biotech) and were used as probes in hybridization. Prehybridization (for 1 h) and overnight hybridization were performed at 68°C in a 1 mM EDTA, 7% (wt/vol) SDS, and 0.5 M Na₂HPO₄ (pH 7.2) solution. Washing steps were performed at room temperature twice in 2× SSC, 0.1% (wt/vol) SDS buffer for 15 min and twice in 0.1× SSC, 0.1% (wt/vol) SDS buffer for 15 min.

### Construction and screening of gene libraries
Partial genomic libraries were constructed from chromosomal DNA of C. butyricum VPI 1718 that was completely digested with restriction enzymes. The 6- to 9-kbp fragment of EcoRI fragments and the 10- to 15-kbp fragment of BglII were agarose purified. Once ligated with EcoRI- or BamHI-digested and dephosphorylated pUC18, the preparation was used to transform competent E. coli DH5α obtained by the method previously described (20). The mixture then was spread on LB agar plates supplemented with ampicillin, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), and isopropyl-β-D-thiogalactopyranoside (IPTG). The transformants were grown overnight at 37°C and then transferred to Hybond-N* membranes (Amersham Pharmacia Biotech) by replica plating, after which they were lysed by alkaline treatment as recommended by the manufacturer. DNA was fixed by being heated at 80°C for 2 h. The membranes then were screened by hybridization as described for Southern blot experiments. Positive clones were tested by restriction analysis and sequencing reactions.

### DNA sequencing
Both strands of DNA were sequenced using the dideoxy chain termination method as described previously (33) with M13 reverse or universal primers or synthetic oligonucleotide primers derived from the sequence obtained from Genome Express (Grenoble, France).

### Determination of the transcription start site
Primer extension reactions were performed as described previously (17), except that superscript reverse transcriptase (Promega) was employed. The dnaPE oligonucleotide (Table 2) complementary to the 5′ end of the dhaD transcript was end labeled with [α-32P]dATP (specific activity, 5,000 Ci/mmol; Amersham Pharmacia Biotech) using T4 polynucleotide kinase (Amersham Pharmacia Biotech). The cDNA was analyzed on an 8% polyacrylamide sequencing gel. To map the exact transcriptional start site, the cDNA was electrophoresed in a lane next to a standard sequencing reaction that was prepared using the same oligonucleotide.

### DNA and amino acid analyses
DNA and amino acid analyses were performed using the Vector NTI program (InVitrogen, Cergy Pontoise, Paris). Sequence comparisons and homology searches were performed using BLAST (1) and PRODOM (8).

### Table 2. Sequences of primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′-3′)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>GBD1</td>
<td>GAAGATCTTGGAATATAAGGAGATTTGATGAG</td>
<td>Forward primer for cloning of dhaD, dhaK, dhaL, and dhaM</td>
</tr>
<tr>
<td>GBD3</td>
<td>TAACCGGATTAATCTAATTTTATTGTTTACCCC</td>
<td>Reverse primer for cloning of dhaD, dhaK, dhaL, and dhaM</td>
</tr>
<tr>
<td>Pat1</td>
<td>AAAAC(A/G)T(T/C)T(A/G)GATA(A/T)GCA(A/T)AAAGC</td>
<td>Reverse primer for the PCR amplification of a dhaD probe</td>
</tr>
<tr>
<td>Pat2</td>
<td>ACA(G/A)/CC(A/G)/TGC(A/T)AACTTTTTC/(A/G)TG</td>
<td>Reverse primer for the PCR amplification of a dhaD probe</td>
</tr>
<tr>
<td>mutH10-D</td>
<td>GATAATGATCGATGTTGTTG</td>
<td>Forward primer for site directed mutagenesis of dhaM</td>
</tr>
<tr>
<td>mutH10-R</td>
<td>CCAATACATGCTGATACTTATAC</td>
<td>Reverse primer for site directed mutagenesis of dhaM</td>
</tr>
<tr>
<td>dhaPE</td>
<td>CTTTACCGTGTACATC</td>
<td>Primer for primer extension analysis of the dhaDKLM operon</td>
</tr>
<tr>
<td>dhaD1-D</td>
<td>ATGAGAAGAACGTATTTGTGGCAAC</td>
<td>Forward primer for northern hybridization of dhaD</td>
</tr>
<tr>
<td>dhaD1-R</td>
<td>GCTTGTACTCTCCTACTTATGACG</td>
<td>Reverse primer for northern hybridization of dhaD</td>
</tr>
<tr>
<td>dhaK3-D</td>
<td>ATGTGTTGAATGGTATAGATGCTACATAG</td>
<td>Forward primer for northern hybridization of dhaM</td>
</tr>
<tr>
<td>dhaK3-R</td>
<td>tTTATTTGATTTCTTATCTCCCTCTACGCG</td>
<td>Reverse primer for dhaDKLM deletion</td>
</tr>
<tr>
<td>dhaKLM-D</td>
<td>ATGGAAAATTTGATCAATGGTGCAAGACGTACCTGG</td>
<td>Forward primer for dhaDKLM deletion</td>
</tr>
<tr>
<td>dhaKLM-R</td>
<td>TTAACCCCGGAGCTGAAAGCTGTCTTATTGAATCTCCCA</td>
<td>Reverse primer for dhaDKLM deletion</td>
</tr>
</tbody>
</table>
Preparation of cell extracts. *C. butyricum* VPI 1718 and *E. coli* extracts were prepared by sonication using the entire anaerobic procedure described previously (40).

Enzyme activity assay. Glycerol dehydrogenase activity was measured by following the procedure described previously (29). The NADH-dependent formation of NAD was spectrophotometrically monitored at 340 nm. The 1-ml assay mixture contained 0.1 mM NADH, 10 U glycerol 3-phosphate dehydrogenase, 100 mM potassium carbonate buffer, pH 9.0, and 100 mM glycerol, and the reaction was initiated by adding the extract.

DHA kinase activity was measured using the procedure described previously (21). NADH consumption was spectrophotometrically monitored at 340 nm using a coupled system in which the DHAP produced by the DHA kinase was reduced by the NADH-dependent glycerol 3-phosphate dehydrogenase. The 1-ml assay mixture contained 1 mM DHA, 1 mM MgCl₂, 0.1 mM NADH, 10 U glycerol 3-phosphate dehydrogenase, 100 mM potassium carbonate buffer, pH 9.0, and 2 mM dithiothreitol. To measure ATP-dependent DHA kinase activity, 1 mM ATP was added. To measure PEP-dependent DHA kinase activity, 1 mM PEP and 100 μM ADP were added. The reaction was started by the addition of DHA.

Nucleotide sequence accession number. The sequence data reported here have been submitted to the GenBank database and assigned accession number AY138581.

RESULTS

Cloning of the DNA region encoding enzymes of the glycerol oxidation pathway. *C. butyricum* VPI 1718 is known to oxidize glycerol through a glycerol dehydrogenase and a DHA kinase (30). Based on highly conserved domains of glycerol dehydrogenases (NH2-gly-gly-gly-lys-thr-leu-asp-thr-alá-lys-ala-COOH [around amino acid residue 100] and NH2-his-gly-glu-lys-val-ala-phe-gly-COOH [around amino acid residue 275]) (9, 11, 24, 38), we designed degenerate oligonucleotides PAT1 and PAT2 (Table 1) to obtain a PCR-amplified 550-bp DNA fragment (pPS1-2). Partial BglII and EcoRI genomic libraries were constructed in pUC18 and screened with pPS1-2 to obtain pPSB10 (10-kb insert) and pSE13 (7.6-kb insert) plasmids, respectively.

Nucleotide sequence analysis. The pPSB10 10,245-bp insert and the pSE13 7,594-bp insert were sequenced. Homology searches of protein and DNA sequence databases allowed the identification of five open reading frames (ORFs) relevant to the glycerol oxidation pathway (Fig. 1A). The ORFs were identified as dhaR (1,032 bp), encoding a putative bacterial transcriptional activator; dhaD (1,140 bp), encoding a putative glycerol dehydrogenase; and dhaK (993 bp), dhaL (627 bp), and dhaM (384 bp), encoding a putative dihydroxyacetone kinase complex. Interestingly, two inverted-repeat sequences followed by a stretch of T residues, resembling a rho-independent terminator, were found between dhaR and dhaD (centered on base 1474; TCATTtTaatAcAATGA; ΔG = -2.4 kcal mol⁻¹) and at the end of the dhaM gene (centered on base 4888; AATGCCTGTAgATATTtaatagATATgTACAGG CATT; ΔG = -13.8 kcal mol⁻¹). The putative genomic arrangement of dhaD, dhaK, dhaL, and dhaM strongly suggests that these genes are organized in an operon.

Amino acid sequence analysis. The amino acid sequences of the proteins encoded by dhaR, dhaD, dhaK, dhaL, and dhaM were deduced. The amino acid sequence of DhaD of *C. butyricum* is approximately 47% identical and 64% similar to the bacterial glycerol dehydrogenases of *C. freundii* (9), *E. coli* (38), *Bacillus stearothermophilus* (24), and *Clostridium beijerinckii* (23).

DhaK amino acid sequence analysis shows high identity to the DhaK subunits of DHA kinases from both *E. coli* (18) and *Lactococcus lactis* subsp. *lactis* (6, 44) (42 and 53% identity, respectively) but low identity to the N-terminal domain of the DHA kinases of *C. freundii* (9) and *Saccharomyces cerevisiae* (25) (38 and 39% identity, respectively).

The DhaL amino acid sequence shows 42% identity to the DhaL subunits of the DHA kinases of *E. coli* (18) and *L. lactis*...
subsp. lactis (6, 44) but only 31 and 32% identity to the C-terminal region of the DHA kinases of C. freundii (9) and S. cerevisiae (25), respectively.

The DhaM amino acid sequence is 40% identical to the DhaM subunit of the DHA kinase of E. coli (18) and 36% identical to the DHA kinase of L. lactis subsp. lactis (6, 44). The sequence analyses of DhaKLM revealed that the C. butyricum DHA kinase is a heteromeric enzyme that consists of three subunits that are similar to the PEP-dependent DHA regulators of E. coli (18) and L. lactis subsp. lactis (44).

The dhaR gene encodes a potential transcriptional regulator. DhaR has a multidomain organization, with N- and C-terminal domains with high similarity to archetypal prokaryotic two-component signal transduction system (TCS) proteins (16, 26). The N-terminal sequence of DhaR (amino acids 1 to 120) has high identity to the N-terminal sensor (or input) domain of the histidine kinase DhaS of Clostridium perfringens (GenBank accession no. NP 561843) and C. butyricum (27, 34) (52 and 46% identity, respectively). The C-terminal domain (amino acids 250 to 344) of DhaR has high identity to the C-terminal output domains of the DhaS-associated DhaA response regulators of C. perfringens (GenBank accession no. NP 561844.1) and C. butyricum (27, 34) (43 and 49% identity, respectively). The C-terminal domain of DhaR contains two helix-turn-helix (HTH) motifs that show sequence similarity to AraC/XylS-type DNA binding proteins found in the TCS response regulator proteins in the other bacteria discussed above (15, 28) (Fig. 2).

The third domain, or intermediate domain (amino acids 130 to 250), has no homology to proteins of known function but contains the five invariant residues of the N-terminal receiver domains of all response regulators characterized thus far: Asp-140, Asp-141, Asp-191, Thr-221, and Lys-242 of DhaR correspond to Asp-12, Asp-13, Asp-57, Thr-87, and Lys-109 of the CheY protein, respectively (22, 41). DhaR homologs are found in the gene cluster of other organisms using this glycerol oxidation system, like Clostridium botulinum (CLL_0536), Paenibacillus larvae (Plarl_010100008220), Bacillus megaterium (Bmqa_1804), and Enterococcus faecalis (Vimssa_355569). However, DhaR of C. butyricum presented no significant homology to DhaR of E. coli.

Transcriptional analysis of the dhaDKLM gene cluster. Northern blot analyses were carried out using total RNA isolated from cells grown in phosphate-limited continuous cultures containing glucose or a mixture of glucose and glycerol as growth substrates (Fig. 1C). The fermentation product profiles of these two cultures were within the experimental errors of previously published data (30). When dhaD or dhaM probe was used, a transcript around 3.2 kb was detected specifically in the glycerol-metabolizing culture. The size of the transcript was in agreement with the polycistronic dhaDKLM operon inferred by sequence analysis. Furthermore, the signal intensities of the 23S and 16S rRNA control bands in the ethidium bromide-stained agarose gels used for the Northern blot analysis were similar for all cultures (Fig. 1C). The Northern blot results were in good agreement with the glycerol dehydrogenase and DHA kinase activities of the corresponding cell extracts (30), demonstrating that the expression of dhaDKLM is regulated at the transcriptional level.

A primer extension study was performed using total RNA extracted from the glycerol-metabolizing continuous culture to identify the transcription start site of the dhaDKLM operon. A unique transcriptional start site (at a C base) corresponding to position 37 upstream of the dhaD start codon was detected (Fig. 1B). The deduced promoter sequence 5'-ATGAGT-3' (−35) and 5'-TATAAT-3' (−10) with a 19-nucleotide (nt) spacing is similar to the α^+ RNA polymerase recognition sequence found in Gram-positive bacteria (43).

Characterization of the glycerol oxidation operon in E. coli. To express the genes encoding the glycerol oxidative pathway of C. butyricum in E. coli constitutively, the dhaDKLM operon first was PCR amplified and cloned into the shuttle vector pSOS95 containing the thiolase promoter from C. acetobutylicum (27), resulting in pVOK1-3. To examine DHA kinase activity without interference from the glycerol dehydrogenase activity of DhaD, the dhaD gene from pVOK1-3 was partially
were introduced into an E. coli strain. Furthermore, crude extracts from E. coli dglA dglpK harboring pVOK1-3, pVOK1-3 ΔD1, or pSOS95 del were assayed for glycerol dehydrogenase activity, and two strains harboring either pVOK1-3 or pSOS95 del were assayed further for DHA kinase activity (Table 3). A glycerol dehydrogenase-specific activity of 288 mU/mg was measured for E. coli dglA dglpK ΔdhaKLM(pVOK1-3), whereas specific activities lower than 1 mU/mg were measured in both E. coli dglA dglpK ΔdhaKLM(pVOK1-3 ΔD1) and E. coli dglA dglpK ΔdhaKLM(pSOS95 del). When PEP was used as a phosphate donor for DHA kinase, a specific activity of 11 mU/mg was measured for E. coli dglA dglpK ΔdhaKLM(pVOK1-3 ΔD1), whereas specific activities lower than 1 mU/mg were measured in E. coli dglA dglpK ΔdhaKLM and E. coli dglA dglpK ΔdhaKLM(pSOS95 del). In contrast, when ATP was used as a phosphate donor for DHA kinase, specific activities lower than 1 mU/mg were measured for both strains. To investigate phosphotransfer in the C. butyricum DHA kinase, two new strains were constructed: E. coli dglA dglpK ΔdhaKLM ΔptsH(pVOK1-3 ΔD1), with a defect in the synthesis of HPr, and E. coli dglA dglpK ΔdhaKLM(pVOK1-3 H9A ΔD1), in which the histidine residue at position 9 of DhaK3 was mutated to alanine residue. The growth of the newly constructed strains was evaluated in M9 DHA liquid medium (together with that of E. coli dglA dglpK ΔdhaKLM(pVOK1-3 ΔD1) and E. coli dglA dglpK ΔdhaKLM(pSOS95 del)) (Fig. 3B). Growth was observed only for E. coli dglA dglpK ΔdhaKLM(pVOK1-3 ΔD1), indicating that HPr and the His9 residue of DhaM are involved in the transfer of a phosphate group from PEP to DHA.

DISCUSSION

In C. butyricum VPI 1718, four genes are involved in the conversion of glycerol into DHAP: dhaD, dhaK, dhaL, and dhaM. The operon arrangement of the four genes, inferred by DNA sequence analysis, was confirmed by Northern blot experiments (Fig. 1C). The dhaDKLM operon was expressed only in the glycerol-metabolizing culture, indicating that this operon is regulated at the transcriptional level. It is likely that the regulation is mediated by the putative regulator DhaR, which is encoded by the dhaR gene located immediately upstream of the operon in the C. butyricum chromosome. DhaR shows significant homology with transcriptional activators from a subgroup of the AraC/XylS family that belongs to the TCS group. These two-component systems usually consist of two different proteins: a sensor protein with a sensor and a trans-mitter domain and a response regulator that possesses a re-

![FIG. 3. Growth curves of different mutants in M9 glycerol and M9 DHA liquid culture. Cultures were performed at 37°C under aerobic conditions in M9 mineral medium supplemented with either 10 mM glycerol (A) or 10 mM DHA (B). ●, E. coli dglA dglpK ΔdhaKLM (pVOK1-3); □, E. coli dglA dglpK ΔdhaKLM ΔdhaR (pVOK1-3 ΔD1); ●, E. coli dglA dglpK ΔdhaKLM ΔptsH (pVOK1-3 ΔD1); ×, E. coli dglA dglpK ΔdhaKLM (pVOK1-3 H9A ΔD1); ▲, E. coli dglA dglpK ΔdhaKLM (pSOS95). OD600, optical density at 600 nm.](http://jb.asm.org/)
FIG. 4. Partial alignments and homology analyses of the glycerol dehydrogenases of C. butyricum, B. stearothermophilus, C. freundii, and E. coli, which all contain the ADH$_{iron-1}$ sequence.

FIG. 5. PEP-dependent DHA kinase of C. butyricum. Shown is a depiction of a working hypothesis of a phosphoryl group transfer from PEP to DHA mediated by the PTS system and the different DhaK subunits. The phosphoryl is transferred sequentially from PEP via enzyme I to H15 of HPr and then to H9 from DhaM, the ADP cofactor of DhaL, and finally to DHA bound to the DhaK subunit. Adapted from Bächler et al. (4).

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