Construction and Characterization of a 1,3-Propanediol Operon

FRANK A. SKRALY, BETSY L. LYTLE,[†] AND DOUGLAS C. CAMERON*

Department of Chemical Engineering, University of Wisconsin—Madison,
Madison, Wisconsin 53706

Received 3 March 1997/Accepted 5 September 1997

The genes for the production of 1,3-propanediol (1,3-PD) in *Klebsiella pneumoniae*, *dhaB*, which encodes glycerol dehydratase, and *dhaT*, which encodes 1,3-PD oxidoreductase, are naturally under the control of two different promoters and are transcribed in different directions. These genes were reconfigured into an operon containing *dhaB* followed by *dhaT* under the control of a single promoter. The operon contains unique restriction sites to facilitate replacement of the promoter and other modifications. In a fed-batch cofermentation of glycerol and glucose, *Escherichia coli* containing the operon consumed 9.3 g of glycerol per liter and produced 6.3 g of 1,3-PD per liter. The fermentation had two distinct phases. In the first phase, significant cell growth occurred and the products were mainly 1,3-PD and acetate. In the second phase, very little growth occurred and the main products were 1,3-PD and pyruvate. The first enzyme in the 1,3-PD pathway, glycerol dehydratase, requires coenzyme B₁₂, which must be provided in *E. coli* fermentations. However, the amount of coenzyme B₁₂ needed was quite small, with 10 nM sufficient for good 1,3-PD production in batch cofermentations. 1,3-PD is a useful intermediate in the production of polyesters. The 1,3-PD operon was designed so that it can be readily modified for expression in other prokaryotic hosts; therefore, it is useful for metabolic engineering of 1,3-PD pathways from glycerol and other substrates such as glucose.

1,3-Propanediol (1,3-PD) is a three-carbon diol that is currently manufactured by synthetic processes beginning with petroleum derivatives such as acrolein or ethylene oxide (35). An emerging large-volume application of 1,3-PD is as a monomer in the synthesis of polyesters for use in carpet and textile fibers (17, 22, 25, 33). Therefore, there is much interest in developing improved routes to 1,3-PD production. One potential method is via the fermentation of glycerol or, ultimately, of sugars (24).

Klebsiella pneumoniae is one of several organisms that naturally ferment glycerol to 1,3-PD. The conversion is carried out in two enzymatic steps. The first enzyme, glycerol dehydratase (EC 4.2.1.30), which requires adenosylcobalamin (coenzyme B₁₂), removes a water molecule from glycerol to form 3-hydroxypropionaldehyde (3-HPA). The second enzyme, 1,3-PD oxidoreductase (EC 1.1.1.202), transfers a reducing equivalent from NADH to 3-HPA, yielding 1,3-PD. The genes encoding glycerol dehydratase and 1,3-PD oxidoreductase are designated *dhaB* and *dhaT*, respectively.

The 1,3-PD pathway was expressed in *Escherichia coli* in our laboratory (41) by using genes from *K. pneumoniae* and in the laboratory of Daniel and Gottschalk (10) by using genes from *Citrobacter freundii*. The main purpose of these endeavors was the characterization of the genes and enzymes responsible for the conversion of glycerol to 1,3-PD. In both cases, the configuration of the 1,3-PD genes and regulatory elements was the same as in the donor organism.

To enhance the utility of the 1,3-PD genes, we investigated their rearrangement into a form that would be adaptable to expression in various hosts under any desired regulation. We sequenced a fragment of *K. pneumoniae* DNA containing the *dhaB* and *dhaT* genes and found that these genes are transcribed in opposite directions. We therefore rearranged the

dhaB and *dhaT* genes into an operon free of the *K. pneumoniae* regulatory elements. In this report, we describe the construction of the 1,3-PD operon and its performance in *E. coli* cofermentations of glycerol and glucose.

This novel genetic configuration provides the basis for an improved microbial 1,3-PD process. Several research groups have achieved 1,3-PD concentrations of 60 to 70 g/liter in the fermentation of glycerol, using organisms that can naturally convert glycerol to 1,3-PD (13, 18, 29, 30). Without directed improvement of the host, however, this level of performance is probably a plateau and cannot compete with newly improved synthetic processes. In 1995, Shell Chemical Company announced an improvement to the ethylene oxide hydrocarbon-ylation process that permits 1,3-PD to be produced at a cost low enough for its use in polypropylene terephthalate carpet fibers (32). Metabolic engineering provides a means to improve the fermentation process. DuPont, for example, recently patented a process to convert sugars to 1,3-PD with various organisms expressing glycerol dehydratase and 1,3-PD oxidoreductase from *K. pneumoniae* (24). The operon we describe in this report is designed so that any promoter and other desired genetic elements can be readily introduced to enable expression of the 1,3-PD genes in various prokaryotes. As such, it should prove useful in the metabolic engineering of 1,3-PD processes.

MATERIALS AND METHODS

DNA sequencing. Sequencing of the *K. pneumoniae* DNA was performed by the dideoxy chain termination method (31) with a Sequenase 2.0 kit (United States Biochemicals, Cleveland, Ohio) at Lofstrand Labs Limited (Gaithersburg, Md.). Two large pieces of DNA to be sequenced, *Apal*-*SacI* and *NheI*-*Apal* fragments, were separately cloned into the vector pSL301 (Invitrogen, San Diego, Calif.). This vector contains a multiple cloning site flanked by T7 and T3 promoter sequences; therefore, T3 and T7 primers were used to initiate double-stranded sequencing. Primer walking with synthetic 18-mer oligonucleotide primers was used to determine the remainder of the double-stranded sequence, with a new primer synthesized for about every 250 nucleotides sequenced.

The high GC content of the sequenced DNA resulted in numerous compressions, which were resolved by the inclusion of 25 or 40% formamide in the sequencing gel or the substitution of 7-deaza-dGTP for dGTP. Artifact banding

* Corresponding author. Mailing address: Department of Chemical Engineering, 1415 Engineering Dr., University of Wisconsin—Madison, Madison, WI 53706-1691. Phone: (608) 262-8931. Fax: (608) 262-5434. E-mail: cameron@engr.wisc.edu.

[†] Present address: Department of Chemical Engineering, University of Rochester, Rochester, NY 14627-0166.

(or shadow bands in all four lanes) was eliminated when it occurred by the addition of terminal deoxynucleotidyl transferase.

Sequence analysis. The Genetics Computer Group (Madison, Wis.) package was used for analysis of open reading frames (ORFs) and restriction sites and for comparisons to GenBank nucleotide sequences. The BLAST server at the National Center for Biotechnology Information (2) was used for amino acid comparisons with the nonredundant protein databases Swiss-Prot, Protein Information Resource, Brookhaven Protein Data Bank, and GenPept.

Bacterial strains, media, and growth conditions. *K. pneumoniae* was obtained from the American Type Culture Collection (Rockville, Md.) as strain ATCC 25955. The *E. coli* strains used in all fermentations were AG1 (Stratagene, La Jolla, Calif.) and TOP10F' (Invitrogen). The temperature for all fermentations and inoculum cultures was 37°C. The volume of the fed-batch fermentation was 4 liters. The volumes of all other fermentations were 300 ml when extracts were prepared or 2 ml when they were not. The fed-batch fermentation was conducted in a Bio-Flo 3000 fermentor (New Brunswick Scientific, Edison, N.J.) with pH controlled at 7.0, agitation at 50 rpm, and nitrogen sparging to minimize dissolved oxygen. Fermentations of the 300-ml total volume were conducted without agitation for at least 10 h in anaerobic flasks. Two-milliliter fermentations were conducted in closed screw-cap tubes with a total liquid volume of 1.8 ml for at least 10 h. Inocula for smaller-scale fermentations were started from stocks frozen in glycerol and grown overnight with shaking in 2 ml of Luria-Bertani medium plus 100 µg of ampicillin per ml (LA medium). The two-milliliter fermentation mixtures were inoculated with 50 µl of the overnight culture, and 300-ml fermentation mixtures were inoculated with 500 µl. The inoculum for the 4-liter fermentation was grown as for the smaller-scale fermentations, and then 100 µl of this culture was added to 50 ml of LA medium and was grown overnight in a closed 50-ml centrifuge tube with no shaking. The entire 50 ml was used to inoculate the 4-liter fermentation.

The standard 1,3-PD production medium was used in all smaller-scale fermentations except where noted otherwise in the text. The standard medium consisted of the following, per liter: 5 g of glycerol, 5 g of glucose, 6 g of Na₂HPO₄, 3 g of KH₂PO₄, 2 g of NH₄Cl, 0.5 g of NaCl, 5 g of yeast extract, 2 mmol of MgSO₄, 100 mg of ampicillin, and 1 to 6 µmol of coenzyme B₁₂. (In initial studies, the standard medium contained 6 µM coenzyme B₁₂, but this was reduced to 1 µM with no apparent effect on the fermentations.) The medium for the 4-liter fermentation was the same as the standard medium, except that 10 g of yeast extract per liter was used, and glucose and glycerol were initially present at 2 g/liter each and were restored to this level when they were consumed completely.

Preparation of cell extracts. Crude cell extracts were prepared by sonication of cell pastes and subsequent centrifugation. Cell pastes were obtained by centrifugation of fermentation broths at 4,000 rpm for 5 to 10 min at 4°C with a Beckman (Fullerton, Calif.) model J2-21 centrifuge and JA-20 rotor. The pastes were washed in 20 mM Tris buffer (pH 8.0) or 50 mM potassium phosphate buffer (pH 8.0), centrifuged as described above, and resuspended in a small amount of the appropriate assay resuspension buffer. The cells were then disrupted by sonication for 5 min on ice at a duty cycle of 70% with 1-s cycles. Cell debris was removed by centrifugation at maximum speed for 5 to 15 min in a microcentrifuge.

Assays. 1,3-PD oxidoreductase was assayed by the method of Johnson and Lin (21). The initial rate of reduction of NAD⁺ to NADH was measured spectrophotometrically (340 nm) at 25°C for a mixture containing 100 mM 1,3-PD, 35 mM ammonium sulfate, 100 mM potassium bicarbonate buffer (pH 9.0), 0.6 mM NAD⁺, and 10 to 50 µl of crude cell extract in a final volume of 1 ml. A baseline was established prior to the addition of NAD⁺. Glycerol dehydrogenase activity was measured by a coupled assay with yeast alcohol dehydrogenase (42) or by the MBTH (3-methyl-2-benzothiazolinone hydrazone) method (43).

For the above assays, 1 U is defined as the number of micromoles of NAD⁺ reduced, NADH oxidized, or propionaldehyde formed per minute at the assay temperature. Total protein concentrations in cell extracts were determined by using the Bradford assay kit (Bio-Rad, Hercules, Calif.) with bovine serum albumin as the standard.

High-performance liquid chromatography analysis. The metabolites present in fermentation broths were analyzed with a Bio-Rad high-performance liquid chromatography system with a refractive index detector and a Bio-Rad Aminex HPX-87H organic acids column at a flow rate of 0.6 ml/min and a column temperature of 65°C. The mobile phase was 0.01 N sulfuric acid. Samples were filtered through 0.45-µm-pore-size Supor membranes (Gelman Sciences, Ann Arbor, Mich.) prior to analysis.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done with a Hoefer (San Francisco, Calif.) SE 600 vertical unit. Each protein sample was diluted with an equal volume of loading buffer (20% [vol/vol] glycerol, 2% [vol/vol] β-mercaptoethanol, 120 mM Tris-Cl [pH 6.8], 41 mg of SDS per ml, 0.01 mg of bromophenol blue per ml) and boiled for 5 min prior to being loaded onto a 1.5-mm-thick gel. The stacking gel and 10% separating gel were prepared as described by Ausubel et al. (4). The gel was run at 20 mA until the blue dye entered the separating gel, and subsequently it was run at 30 mA for 3 h.

Construction of plasmids. Standard techniques of recombinant DNA technology as described by Ausubel et al. (4) were used for all DNA manipulations. Restriction and DNA-modifying enzymes were obtained from Promega (Madi-

TABLE 1. Cloning vectors used and plasmids cited in the text

Plasmid	Properties	Source or reference
pBR322	<i>E. coli</i> cloning vector	9
pSE280	<i>trc</i> promoter upstream of MCS ^a	Invitrogen
pSE380	pSE280 with constitutive <i>lac</i> repressor gene downstream of MCS	Invitrogen
pSL301	<i>lac</i> promoter upstream of MCS	Invitrogen
PTC1	Cosmid containing <i>K. pneumoniae dhaB</i> , <i>dhaT</i> , <i>dhaD</i> , and <i>dhaK</i> genes	41
PTC3	12.1-kb <i>NheI</i> - <i>SacI</i> fragment of pTC1 inserted into pBR322	39
PTC9	230-bp <i>EcoRI</i> - <i>NheI</i> fragment deleted from pTC3 and remaining <i>HindIII</i> site within <i>K. pneumoniae</i> DNA filled in	39
PTC42	2.3-kb fragment of pTC9 from start of ORF 2 (<i>dhaT</i>) to <i>SacI</i> site inserted into pSE380	This work
PTC48	1.3-PD operon; unidirectional transcription of <i>dhaB</i> and <i>dhaT</i> from <i>trc</i> promoter; construction described in Materials and Methods	This work
PTC49	pTC48 with 596-bp <i>SalI</i> - <i>NotI</i> fragment replaced by 677-bp PCR-derived ORF 4 upstream region	This work
PTC50	pTC48 with 596-bp <i>SalI</i> - <i>NotI</i> fragment replaced by 603-bp PCR-derived ORF 4 upstream region	This work
PTC53	pTC50 with <i>lacI</i> ^q gene inserted at <i>SacI</i> site	This work
PTC63	1.5-kb <i>NruI</i> - <i>NruI</i> fragment (within ORF 3) deleted from pTC49	This work
pTrcB1	5.3-kb <i>KpnI</i> - <i>NheI</i> fragment of pTC9 inserted into pSE280	44

^a MCS, multiple cloning site.

son, Wis.) or New England Biolabs (Beverly, Mass.) and used according to the instructions of the manufacturer. Plasmids used in experimentation are described in Table 1. Some plasmids that were used only in construction are not listed in Table 1 but are mentioned below.

The prototype operon (pTC48) was constructed by the following steps, as illustrated in Fig. 1. The source of the *dhaB* and *dhaT* genes was pTC9 (39). A partial *dhaT* gene was isolated by PCR so that the product had a 5' *KpnI* site followed by the naturally occurring 17 nucleotides upstream of the *dhaT* gene and a sufficient length of the structural *dhaT* gene to include the internal *PstI* site. This product was cut with *KpnI* and *PstI* and inserted into pSE380 (Invitrogen) cut with the same two enzymes to give pTC35. pTC35 was cut with *PstI* and *SacI*, both originally in the multiple cloning site of pSE380, so that the remainder of the *dhaT* gene (the *PstI*-*SacI* fragment of pTC3) could be inserted. The resulting plasmid contained the full *dhaT* gene under the control of the *trc* promoter. The *trc* promoter allows high-level expression inducible by isopropyl-β-D-thiogalactoside (IPTG). A partial *dhaB* gene was isolated by PCR so that the product had a 5' *SalI* site followed by the nucleotide sequence GAGGTAACAAAG and a sufficient length of the structural *dhaB* gene to include the internal *NotI* site. This product was cut with *SalI* and *NotI* and inserted into pTC3 cut with the same two enzymes to give pTC43, which contains a full promoterless *dhaB* gene and none of the other *dha* genes of *K. pneumoniae*. pTC44 was constructed by cutting both pTC42 and pTC43 with *NheI* and *SalI*, ligating the two products, and isolating the plasmid which contained both the *dhaB* and the *dhaT* genes transcribed in the same direction under the control of the *trc* promoter. Extraneous start codons were present in pTC44, however, and therefore the *trc* promoter was isolated from pSE380 by PCR so that it could be placed before the *dhaB* and *dhaT* genes with no start codons between itself and the start codon of *dhaB*. The *trc* PCR product was flanked by *NdeI* and *SalI* sites, and these enzymes were used to cut the *trc* PCR product as well as pSL301. pTC46S was the ligation product of these two fragments, such that the *trc* promoter was introduced into the multiple cloning site of pSL301. pTC46S and pBR322 were both cut with *NdeI* and *SalI* and ligated together so that the product, pTC47A, contained the *trc* promoter and the ampicillin resistance gene and replication origin of pBR322. pTC47A and pTC44 were both cut with *SalI* and *HindIII* so that the *trc* promoter was just upstream of the *dhaB*-*dhaT* region. pTC48 enabled the conversion of glycerol to 1,3-PD in *E. coli*.

Two derivatives of pTC48 (pTC49 and pTC50) were constructed by replacing the *SalI*-*NotI* fragment of pTC48 with the products of PCRs using pTC9 as a template. The same downstream primer within ORF 4 that was used for pTC48 was used to generate these two products, but the upstream primer was made to

FIG. 1. Construction of pTC48 as outlined in Materials and Methods. Restriction enzyme recognition sites are abbreviated as follows: H, *Hind*III; K, *Kpn*I; Nd, *Nde*I; Nh, *Nhe*I; No, *Not*I; P, *Pst*I; Sc, *Sac*I; and SI, *Sal*I. Brackets indicate the combination of two fragments of DNA by cleavage at the restriction sites shown in the bracket and subsequent ligation to form the product shown beneath the bracket.

hybridize to regions of pTC9 further upstream of ORF 4 than the primer used in the construction of pTC48. pTC53 is identical to pTC50 except that the constitutive *lac* repressor, isolated by PCR from the vector pSE380, was inserted into the *Sac*I site so that the *lac* repressor is transcribed in the same direction as the 1,3-PD genes.

pTrcB1 was constructed by ligating the *Kpn*I-*Nhe*I fragment of pTC9 into pSE280 (Invitrogen) cut with *Kpn*I and *Spe*I.

Nucleotide sequence accession number. The nucleotide sequence described in this paper was submitted to GenBank under accession no. U30903.

RESULTS

General features of the DNA sequence. The sequence of an *Nhe*I-*Sac*I fragment of cosmid pTC1 (41), a contiguous sequence of 8,067 nucleotides, was determined as described in Materials and Methods. Several large ORFs (that would encode proteins of 10 kDa or more) were found within the fragment known to contain the *dhaB* and *dhaT* genes. The major ORFs were designated as shown in Fig. 2.

Identification of functional units conferring *dhaB* and *dhaT* activities. The main objective in the construction of the 1,3-PD operon was to enable expression of the *dhaB* and *dhaT* genes in one transcript under the regulation of a single replaceable promoter/operator. The construction of the 1,3-PD operon therefore required the identification of regions of DNA that confer *dhaB* and *dhaT* activities but are independent of their native regulation.

Glycerol dehydratase (DhaB) is known to consist of multiple

subunits. This suggests that the DNA encoding it consists of multiple ORFs. Stroinski et al. (34) reported that the active DhaB enzyme consists of two subunits, A (22 kDa), which itself dissociates into two subunits with an apparent molecular mass of about 12 kDa each, and B (189 $\pm$ 22 kDa). It was shown that subunit B could be further dissociated into subunits of 90 $\pm$ 25 kDa in the presence of 0.1 M KCl. A portion of the sequenced fragment, the *Kpn*I-*Nhe*I fragment, was found to be sufficient for *dhaB* activity. This fragment contains ORFs transcribed in

FIG. 2. Major ORFs within the sequenced *K. pneumoniae* DNA fragment. The direction of transcription is indicated (arrows). The three rows shown for each transcriptional direction represent the three frames of reference. ORFs incorporated into the 1,3-PD operon are indicated (shaded). Glycerol dehydratase is encoded by ORFs 4, 4a, and 3a. 1,3-PD oxidoreductase is encoded by ORF 2. ORF 3 augments 1,3-PD production by providing an unknown function.

the following order: 4, 4a, 3a, 3. Plasmid pTrcB1 contains the *KpnI-NheI* fragment under control of the *trc* promoter. Crude cell extracts of *E. coli*/pTrcB1 possessed glycerol dehydratase activity. Living *E. coli*/pTrcB1 grown in the standard medium, except with glucose and glycerol replaced by xylose and 1,2-propanediol (1,2-PD), converted 1,2-PD to 1-propanol only when coenzyme B₁₂ was added to the medium. *K. pneumoniae* glycerol dehydratase can accept 1,2-PD as a substrate, and the conversion of 1,2-PD to 1-propanol is analogous to the conversion of glycerol to 1,3-PD. 1,2-PD was used in place of glycerol to avoid accumulation of highly toxic 3-HPA, and xylose was used instead of glucose to avoid possible catabolite repression of the endogenous dehydrogenase responsible for conversion of propionaldehyde to 1-propanol.

1,3-PD oxidoreductase (DhaT) is reported (12, 21) to consist of a single subunit of 40 to 45 kDa whose active form comprises an octamer of this subunit. We cloned ORF 2 downstream of the *trc* promoter and included a copy of the constitutive *lac* repressor gene to form plasmid pTC42. We compared *dhaT* activity levels in extracts of induced and uninduced *E. coli*/pTC42 cells. We also compared these to the activities in extracts of *E. coli* cells containing pTC9, a plasmid that allows significant 1,3-PD synthesis, to ensure that pTC42 was capable of conferring adequate *dhaT* activity. When pTC42 fermentation cultures were uninduced, the resulting cell extracts possessed no detectable *dhaT* activity. pTC42 fermentation mixtures that contained 0.5 mM IPTG throughout gave cell extracts with a *dhaT* activity (0.8 U/mg of protein) twice that of pTC9 cell extracts (0.4 U/mg of protein). ORF 2 was expected to encode a protein with a molecular weight of 41,459 based on translation of the nucleotide sequence. SDS-PAGE of induced and uninduced pTC42 extracts showed that induced cells overproduced a protein of just under 43 kDa, whereas uninduced cells did not (Fig. 3).

Comparison of identity at the amino acid level provided further evidence that ORF 2 encodes 1,3-PD oxidoreductase. ORF 2 is homologous to a number of NADH-dependent oxidoreductases having identities at the amino acid level of from 36% with *E. coli* alcohol:NAD⁺ oxidoreductase (AdhE) to 94% with *C. freundii* 1,3-PD:NAD⁺ oxidoreductase (DhaT). Daniel et al. (11) have reported the homology of the *C. freundii* DhaT protein to a number of type III oxidoreductases. The gene product of ORF 2, like the type III oxidoreductases, contains the characteristic iron-containing-protein signature GxxHxxAHxxGxxxxPHG (5).

The above evidence led to the assignment of the regions of DNA to be considered functional *dhaB* and *dhaT* units for the purpose of operon construction. For *dhaB*, the unit is the set of ORFs to be transcribed in the order 4, 4a, 3a, 3. For *dhaT*, the unit is ORF 2.

Construction of the 1,3-PD operon. In the native DNA fragment isolated from *K. pneumoniae* the *dhaT* and *dhaB* genes are transcribed in opposite directions from a common region. In Fig. 2, ORF 2 (*dhaT*) is transcribed from left to right, and ORFs 4, 4a, 3a, and 3 are transcribed from right to left. Therefore, the steps necessary for construction of the 1,3-PD operon were (i) isolation of the functional *dhaB* and *dhaT* units without their promoters, (ii) rearrangement of the DNA so that the two units are transcribed in the same direction, and (iii) attachment of a replaceable promoter upstream of the units. A representation of the 1,3-PD operon (plasmid pTC49) is shown in Fig. 4.

Construction of the 1,3-PD operon was accomplished by PCR, restriction digestions, and ligations as described in Materials and Methods. ORFs 2 and 4 were amplified by PCR from the beginning of the ORF to a site downstream of an

FIG. 3. Coomassie blue-stained SDS-PAGE gel of crude cell extracts of *E. coli*/pTC42, induced with 0.5 mM IPTG (lane +) and uninduced (lane -). The protein overproduced when the cells are induced is indicated (arrow). Crude cell extracts were prepared as described in Materials and Methods. Lane M, molecular mass marker.

internal restriction site and religated to the remainder of the corresponding gene. The *trc* promoter (with the *lac* repressor binding site but no catabolite gene activator protein-cyclic AMP binding site) was isolated by PCR from pSE380 so that the amplification product would be bounded by *Bgl*II sites. The promoterless genes and the *trc* promoter were reassembled into pBR322 so that the *trc* promoter directs monocistronic transcription of the ORFs in the order 4, 4a, 3a, 3, 2.

The significance of ORF 3. Tobimatsu et al. (38) reported that ORF 3 does not encode a subunit of glycerol dehydratase. We observed a loss of dehydratase activity when the *Sfi*I site within ORF 3 of pTC9 was disrupted by digestion and blunt-end ligation (44). The relevance of ORF 3 to the 1,3-PD operon was tested by conducting fermentations with strain TOP10F' carrying either pTC49 or pTC63 (pTC49 with an *Nru*I-*Nru*I deletion within ORF 3). TOP10F'/pTC63 produced about 40% as much 1,3-PD as TOP10F'/pTC49 (Table 2), indicating that ORF 3 is not necessary for glycerol dehydratase activity, but it may serve some other function that permits 1,3-PD synthesis to be more effective.

Requirement for coenzyme B₁₂ addition. We determined to what extent coenzyme B₁₂ addition was necessary to effect 1,3-PD production in the transgenic *E. coli* cells. Figure 5 shows the final 1,3-PD concentrations of fermentations in which TOP10F'/pTC49 was grown in the standard 1,3-PD production medium except with various low concentrations of coenzyme B₁₂. 1,3-PD was not formed by TOP10F'/pTC49 when coenzyme B₁₂ was not added, and TOP10F' cells did not synthesize 1,3-PD without the *K. pneumoniae* genes, confirming that the

FIG. 4. Plasmid pTC49, one version of the 1,3-PD operon. The regions sufficient for glycerol dehydratase (*dhaB*) and 1,3-PD oxidoreductase (*dhaT*) expression (black segments) and the direction of transcription (arrows) are indicated. The *ampR* (ampicillin resistance) gene and the pMB1 origin of replication are identical to those in pBR322. Nucleotide positions are also indicated (in parentheses).

cloned *K. pneumoniae* genes are responsible for 1,3-PD synthesis. Coenzyme B₁₂ was no longer limiting to 1,3-PD synthesis when provided at concentrations above 10 nM, far below the concentration in our standard production medium (1 μ M).

Fed-batch fermentation. We conducted a 4-liter fed-batch cofermentation of glycerol and glucose with *E. coli* AG1/pTC53 without induction to determine what concentration of 1,3-PD could be achieved in the final broth. pTC53 is essentially the same as pTC49 except that it contains the constitutive *lac* repressor gene (*lacI^q*). pTC49, although it can be somewhat more effective in 1,3-PD synthesis, was not used because of its tendency to rearrange. pTC49 and pTC53 are stable plasmids, but pTC49 often did not retain its original configuration over many generations. It is possible that the addition of the *lacI^q* gene to pTC49 prevents excessive transcription and that rearrangement in pTC53 does not provide a significant growth advantage. Figure 6 shows the time course of the AG1/pTC53 fermentation, in which glucose and glycerol were adjusted to 2 g/liter each time the concentrations of both components were nearing zero. The results of the fermentation are summarized in Table 3. The final concentration of 1,3-PD was 6.3 g/liter. The fermentation had two distinct phases, one in which the

cells grew and produced predominantly 1,3-PD and acetate, and another after the cells ceased to grow in which pyruvate became the dominant acid product.

DISCUSSION

We have constructed an operon containing the glycerol dehydratase and 1,3-PD oxidoreductase genes of *K. pneumoniae*.

TABLE 2. Production of 1,3-PD in batch culture by TOP10F'/pTC49 and TOP10F'/pTC63^a

Initial glycerol concn (g/liter)	1,3-PD produced (g/liter)	
	TOP10F'/pTC49	TOP10F'/pTC63
2	0.96	0.43
5	1.19	0.49
10	1.18	0.47

^a Fermentations were carried out in the standard 1,3-PD production medium in closed 2-ml screw-cap tubes with no agitation for 15 h at 37°C. pTC63 is identical to pTC49 except that it lacks the *NruI*-*NruI* fragment (1.5 kb) within ORF 3.

FIG. 5. 1,3-PD production in *E. coli* TOP10F' carrying pTC49. The 2-ml fermentations were carried out as described in Materials and Methods in standard 1,3-PD production medium but with various low concentrations of coenzyme B₁₂. Symbols: □, final 1,3-PD concentration (in grams per liter); ■, molar yield of 1,3-PD from glycerol (moles of 1,3-PD produced per mole of glycerol consumed).

FIG. 6. Time course of the 4-liter fed-batch fermentation of *E. coli* AG1 carrying pTC53. (A) Substrate consumption, growth, and 1,3-PD production. Concentrations in the fermentor are shown for glycerol (■), glucose (□), and 1,3-PD (●), as well as the optical density of cells at 660 nm (○). (B) By-product formation. Concentrations in the fermentor are shown for acetate (▲), formate (○), lactate (□), pyruvate (■), and succinate (●).

The operon enables the production of 1,3-PD in *E. coli* and provides the basis for future expression of the 1,3-PD pathway under novel regulation in other organisms. It also provides a basis for extending the 1,3-PD pathway to include fermentable sugars. Laffend et al. (24) have already demonstrated that the sugars-to-1,3-PD pathway is realizable. Expression of the 1,3-PD genes in an organism that can naturally produce glycerol from sugars would complete a microbial sugars-to-1,3-PD pathway, averting the possibly toxic effects of high glycerol concentrations and providing a microbial route to 1,3-PD from fermentable sugars, which are more abundant, less expensive, and utilizable by a wider range of organisms than glycerol.

The 1,3-PD operon is very flexible in that it contains unique restriction sites in several strategic locations (Fig. 4). The promoter region can be replaced with any *Bgl*II-*Bgl*II fragment; PCR can be used to isolate any promoter region of interest bounded by *Bgl*II sites, and the promoter region can be ligated into this location. We observed that the *trc* promoter fragment could be replaced by the *E. coli lac* or *phoA* promoter with the retention of the ability to produce 1,3-PD, but removal of the promoter caused a dramatic decrease in 1,3-PD production (data not shown). The *Bgl*II sites flanking the promoter are also significant because DNA cleaved with *Bgl*II can be ligated to DNA cleaved with *Sau*3AI without the creation of blunt ends with DNA polymerase. Therefore, *Sau*3AI digests of genomic DNA of any organism can be randomly ligated into this location to screen for promoters effective in the production of 1,3-PD under any conditions desired, an implementation not possible with unmanipulated *K. pneumoniae* DNA. The upstream regions of ORF 2 or 4 can be replaced with any *Kpn*I-*Mlu*I or *Sal*I-*Not*I fragment, respectively. This allows the introduction of different ribosome binding sites or leader sequences at either of these locations. Additional genes, promoters, and terminator structures can be ligated into the operon before or after the promoter, dehydratase region, or oxidoreductase region.

During the construction of the 1,3-PD operon, we determined that ORFs 4, 4a, 3a, and 3 are sufficient for glycerol dehydratase activity. Two important points concerning this determination should be addressed here: the demonstration of

the activity and the significance of ORF 3. We observed that cells containing pTrcB1 (ORFs 4, 4a, 3a, and 3 under control of the *trc* promoter) possessed glycerol dehydratase activity. These cells converted 1,2-PD to 1-propanol when the medium was supplemented with coenzyme B₁₂. Glycerol dehydratase converts glycerol to 3-HPA, which is a potent antimicrobial agent (15, 36), but it converts 1,2-PD to propionaldehyde, which is less toxic to *E. coli* than is 3-HPA (15, 23). We therefore used 1,2-PD as a substrate instead of glycerol to demonstrate the activity in whole cells.

Tobimatsu et al. (38) reported that the *K. pneumoniae* ORF corresponding to our ORF 3 is not necessary for glycerol dehydratase activity. We found this to be true also, but we found that ORF 3 seems to augment 1,3-PD synthesis by providing an unknown function. ORF 3 has some identity to coenzyme B₁₂-dependent enzymes. A generally recognized conserved sequence in such enzymes is DxHxxG (7). This motif is not encoded in ORF 3; however, the translation of ORF 3 shares a VGxSSL motif with the coenzyme B₁₂-dependent enzymes methylmalonyl-coenzyme A mutase (8, 28), methy-

TABLE 3. Summary of results of the 4-liter fed-batch *E. coli* AG1/pTC53 fermentation

Parameter	Phase I (0–8 h)	Phase II (8–32 h)	Overall (0–32 h)
Glycerol consumed (g/liter)	1.85	7.48	9.33
Glucose consumed (g/liter)	2.00	6.70	8.70
Yield (mol of 1,3-PD/mol of glycerol)	0.77	0.83	0.82
Products formed (g/liter)			
1,3-PD	1.18	5.15	6.33
Acetate	1.09	0.95	2.04
Succinate	0.32	0.11	0.43
Pyruvate	0.00	4.11	4.11
Lactate	0.29	1.35	1.64
Formate	0.79	0.65	1.44
Biomass	0.69	0.06	0.75
Carbon recovery (%)	105	92	95

leneglutamate mutase (7), glutamate mutase (27), and methionine synthase (6). This motif, however, does not occur in all coenzyme B₁₂-dependent enzymes, as it is not contained in ethanolamine ammonia-lyase (14, 16). The similarity of ORF 3 to several genes encoding coenzyme B₁₂-dependent enzymes suggests that the gene product of ORF 3 may interact with coenzyme B₁₂ or perhaps with the glycerol dehydratase holoenzyme.

A 4-liter fed-batch fermentation culture with *E. coli* AG1 carrying plasmid pTC53 accumulated 6.3 g of 1,3-PD per liter. This is about the same level as in a fermentation with AG1/pTC9, in which 6.5 g of 1,3-PD accumulated per liter (39). pTC9 contains the *K. pneumoniae* 1,3-PD genes in an unaltered form. Therefore, the 1,3-PD operon has retained the ability to effectively direct 1,3-PD synthesis while having the advantage that it can be expressed in any prokaryotic organism, provided that an appropriate promoter is inserted and, if necessary, that the ribosome binding sites are adjusted. As *dhaT* is an octamer of a single subunit (ORF 2), it may be advantageous in the future to optimize the ratio of *dhaB* to *dhaT* expression in the operon, perhaps by providing ribosome binding sites of various strengths or even a second promoter upstream of *dhaT*. The flexible construction of the 1,3-PD operon allows such changes to be made readily.

Theoretically, 1 mol of 1,3-PD can be produced for every mol of glycerol consumed, given that the cells are provided with a cosubstrate such as glucose from which to derive reducing power (40). We observed a yield of 82% in the AG1/pTC53 fermentation, which means that 18% of the glycerol consumed was not converted to 1,3-PD. In the absence of respiration, *E. coli* cannot use glycerol as the sole source of carbon and energy because neither of the two known *sn*-glycerol-3-phosphate dehydrogenases of *E. coli* can use NAD⁺ as an electron acceptor (26). One of these dehydrogenases can donate electrons to the fumarate reductase complex, which produces succinate from fumarate, but the quantity of succinate produced in the AG1/pTC53 fermentation could account for the metabolism of only about 4% of the glycerol in this manner. Biomass cannot account for the disparity, because in the second phase of the fermentation (Table 3), very little biomass was produced while most of the glycerol was consumed, and the molar yield of 1,3-PD was 83%. Therefore, it may be that background activities resembling glycerol dehydrogenase (3, 20) and dihydroxyacetone kinase (19) are responsible for the conversion of a small portion of the glycerol to dihydroxyacetone phosphate, a glycolytic intermediate.

E. coli with the 1,3-PD operon gives a product distribution not previously observed in 1,3-PD fermentations. Pyruvate accumulated during 1,3-PD synthesis after the AG1/pTC53 cells in the 4-liter fermentation culture had ceased to grow. Presumably, this is because the enzymes of anaerobic pyruvate dissimilation are repressed somewhat when *E. coli* enters stationary phase. Pyruvate accumulation has been observed in *E. coli* fermentations where glucose consumption exceeds the growth requirement (37). 1,3-PD fermentations must yield some side product, usually predominantly acetate, in addition to 1,3-PD so that NADH can be regenerated. The pathways from glucose to either acetate or pyruvate have the same NADH yield, but the pyruvate pathway yields half as much ATP as the acetate pathway. Acetate is a main by-product of the AG1/pTC53 fermentation while the cells are growing and require more ATP, but pyruvate becomes the dominant acid product when growth ceases and the ATP requirement decreases.

Overexpression of the 1,3-PD genes in organisms other than *E. coli* may enable improved 1,3-PD production. For example,

this could be accomplished by expressing the 1,3-PD operon in natural producers of 1,3-PD or in organisms highly tolerant to 1,3-PD. It has been shown elsewhere (1) that glycerol dehydratase activity is the limiting factor in 1,3-PD production in *Clostridium butyricum*. Therefore, increasing the dehydratase activity in such an organism could increase the productivity of the 1,3-PD fermentation. *K. pneumoniae*, a natural 1,3-PD producer, can no longer grow in the presence of about 70 g of 1,3-PD per liter (39). This is also approximately the highest 1,3-PD concentration reported for a *K. pneumoniae* fermentation (18). The mechanism of the inhibition is not well understood. If the inhibition is not specific to the 1,3-PD pathway expression of the operon in *K. pneumoniae* could increase the rate of production, but not the final concentration, of 1,3-PD. Expression of the operon in organisms with greater tolerance of 1,3-PD could lead to higher concentrations of 1,3-PD than are currently possible.

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