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Propanediol Utilization Genes (*pdu*) of *Salmonella typhimurium*: Three Genes for the Propanediol Dehydratase†

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The propanediol utilization (*pdu*) operon of *Salmonella typhimurium* encodes proteins required for the catabolism of propanediol, including a coenzyme B_{12}-dependent propanediol dehydratase. A clone that expresses propanediol dehydratase activity was isolated from a *Salmonella* genomic library. DNA sequence analysis showed that the clone included part of the *pduF* gene, the *pduABCDE* genes, and a long partial open reading frame (ORF1). The clone included 3.9 kbp of *pdu* DNA which had not been previously sequenced. Complementation and expression studies with subclones constructed via PCR showed that three genes (*pduCDE*) are necessary and sufficient for propanediol dehydratase activity. The function of ORF1 was not determined. Analyses showed that the *S. typhimurium* propanediol dehydratase was related to coenzyme B_{12}-dependent glycerol dehydratases from *Citrobacter freundii* and *Klebsiella pneumoniae*. Unexpectedly, the *S. typhimurium* propanediol dehydratase was found to be 98% identical in amino acid sequence to the *Klebsiella oxytoca* propanediol dehydratase; this is a much higher identity than expected, given the relationship between these organisms. DNA sequence analyses also supported previous studies indicating that the *pdu* operon was inherited along with the adjacent cobalamin biosynthesis operon by a single horizontal gene transfer.

Salmonella catabolizes propanediol by a pathway that employs the vitamin B_{12} coenzyme, adenosylcobalamin (Ado-CBL). Several lines of evidence signify the importance of cobalamin-dependent propanediol utilization to the *Salmonella* lifestyle. The propanediol utilization (*pdu*) genes and the cobalamin synthetic genes (*cob*) are contiguous and are coinduced by propanediol, indicating that propanediol catabolism is the primary reason for the de novo synthesis of the vitamin B_{12} coenzymes by *Salmonella* (1, 9, 34). If one includes the *cob* genes, *Salmonella* maintains 40 to 50 genes primarily for the transformation of propanediol (12, 22, 24, 36). Of 138 natural *Salmonella* isolates (the SARA and SARB collections), at least 134 synthesize vitamin B_{12} de novo under anaerobic conditions and at least 129 degrade propanediol (25). The combination of propanediol degradation and vitamin B_{12} synthesis is the basis of one criterion for the identification of *Salmonella* (33). These aspects of *Salmonella* metabolism have been reviewed recently (35).

Still, the use of propanediol by *Salmonella* presents a paradox. Propanediol is formed by the decomposition of rhamnose and fucose (5). These sugars are common constituents of plant cell walls and of the glycoconjugates of intestinal epithelial cells (27). Hence, *Salmonella* would be expected to encounter propanediol in the gut. Consistent with this expectation, *Salmonella* synthesizes the vitamin B_{12} coenzymes de novo only under anaerobic conditions (23). However, the contribution of propanediol to growth in anaerobic environments is uncertain. *Salmonella* cannot use propanediol as the sole carbon and energy source under standard anaerobic laboratory conditions even if provided with alternative electron acceptors such as nitrate, fumarate, trimethylamine *N*-oxide, or dimethyl sulfoxide (8). The less well-studied terminal electron acceptor, tetrathionate, does support the anaerobic use of propanediol (8), but the availability of tetrathionate and other polysulfides in the natural environments of *Salmonella* is uncertain (6). Propanediol also serves as a sole carbon and energy source aerobically, but in the presence of oxygen *Salmonella* is unable to synthesize Ado-CBL de novo (22, 23, 25). Aerobic growth on propanediol relies on exogenous Ado-CBL or a complex precursor such as cyanocobalamin (CN-CBL; vitamin B_{12}).

The pathway of propanediol catabolism by *Salmonella typhimurium* has been investigated (32, 43). Anaerobically, propanediol is converted to propionaldehyde and then to equal amounts of propanol and propionic acid. The enzymes of the propanediol catabolic pathway include an Ado-CBL-dependent propanediol dehydratase that converts propanediol to propionaldehyde (26). The remaining pathway enzymes, which convert propionaldehyde to propionic acid and propanol, are proposed to be coenzyme A (CoA)-dependent aldehyde dehydrogenase, phoshophotransacylase, propionate kinase, and alcohol dehydrogenase (32, 43). Overall, propanediol catabolism provides one ATP molecule per propanediol molecule but no source of carbon. Aerobically, propanediol can provide both carbon and energy when exogenous vitamin B_{12} is provided (22). The aerobic and anaerobic pathways of propanediol catabolism are probably similar, but when oxygen is available some propionyl-CoA can be diverted to central metabolism via the propionate (*prp*) pathway (21, 43).

Genes involved in propanediol utilization cluster at the *pdu* locus (Fig. 1). Genetic studies have shown that this locus is very large, about 20 kbp in length (8, 24). The *pocR* and *pduF* genes encode a positive regulatory protein and a propanediol diffusion facilitator, respectively (9, 12, 34). The *pdu* operon is thought to encode all the enzymes of the propanediol catabolic pathway (8, 22). Thus far, *pdu* mutants that lack Ado-CBL-dependent propanediol dehydratase activity and CoA-dependent propionaldehyde dehydrogenase activity have been iso-

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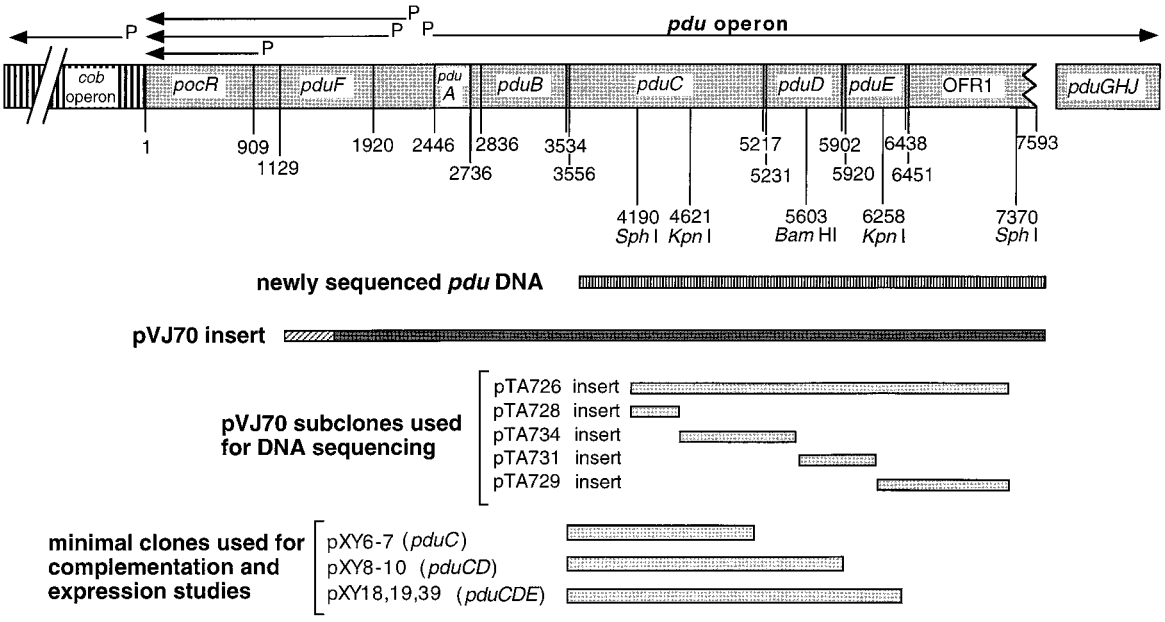

FIG. 1. *S. typhimurium pdu* locus. Genes identified by DNA sequence analysis are shown (12, 36; see the text). See GenBank sequence AF026270. The start and stop sites of coding regions of the *pdu* genes are indicated. The *pduGHJ* genes, which were identified by genetic tests, are also shown (45). These genes have not been correlated with the physical map. Genetic studies place them in the *pdu* operon somewhere downstream of the *pduE* gene (45). The DNA sequence determined in this study is diagrammed, as are the cloned inserts used as a sequencing template. The locations of restriction sites used in subcloning are shown. Also diagrammed are the minimal clones used for complementation and expression studies; these were constructed by cloning PCR products. The currently known promoters of the *pdu* locus are shown as arrows at the top of the figure.

lated (22, 45). Recent genetic studies suggest that the *pdu* operon also contains at least one gene (*pduG*) needed for the conversion of CN-CBL to Ado-CBL (45). In addition, the *pdu* operon includes a gene (*pduA*) that is homologous to those that encode the subunit proteins of carboxysomes, polyhedral vesicles that compartmentalize ribulose bisphosphate carboxylase/monooxygenase (Rubisco) in some chemo- and photoautotrophs (12). The *pduA* gene probably encodes a component of the polyhedral bodies that *S. typhimurium* forms during anaerobic growth in the presence of propanediol (8). Two additional cistrons (*pduHJ*), identified by complementation analysis, have unknown functions (45). Considering the estimated size of the *pdu* operon (20 kbp), it probably contains 5 to 10 genes in addition to those referred to above (8, 24).

Here we report the DNA sequence of 3.9 kbp of the *pdu* operon. The new sequence (Fig. 1) completes the *pduC* gene, includes two long complete open reading frames (ORFs) (*pduDE*) and a long incomplete open reading frame (ORF1). The *pduCDE* genes are shown to be necessary and sufficient for propanediol dehydratase activity. The function of ORF1 was not determined. Analyses show that the *S. typhimurium* propanediol dehydratase is nearly identical to the *Klebsiella oxytoca* propanediol dehydratase and is related to Ado-CBL-dependent glycerol dehydratases from *Citrobacter freundii* and *Klebsiella pneumoniae*. DNA sequence analyses also support previous work indicating that the *pdu* operon was inherited along with the nearby *cob* operon by a single horizontal gene transfer (25, 36).

MATERIALS AND METHODS

Chemical reagents and enzymes. Isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) were from Diagnostic Chemicals Ltd., Charlottetown, Canada. Alcohol dehydrogenase and (*S*)-1,2-propanediol were from Boehringer Mannheim (Indianapolis, Ind.); (*R,S*)-1,2-propanediol was from Aldrich Chemical Co., Milwaukee, Wis. Restriction enzymes and T4 DNA ligase were from New England Biolabs, Beverly, Mass. Vitamin B_{12} and other chemicals were from Sigma Chemical Co., St. Louis, Mo.

Bacterial stains, media, and growth conditions. The bacterial strains used in this study are listed in Table 1. The minimal medium used was NCE medium (7, 44) supplemented with 1% disodium succinate, 1 mM $MgSO_4$, 0.1 mM histidine, and 0.3 mM methionine. Luria-Bertani (LB) medium was the rich medium used

TABLE 1. Bacterial strains used in this study

Strain	Genotype
E. coli	
DH5α	F$^-$ λ$^-$ *endA1 hsdR17 relA1 supE44 thi-1 recA1 gyrA96 relA1* Δ(*lacZYA-argF*)*U169* (ϕ80d*lacZ*Δ*M15*)
JM107	Δ(*lac-proAB*) *thi gyrA96 endA1 hsdR17 relA1 supE44*/F′ *traD36 lacI*^{q} Δ(*lacZ*)*M15* ProA$^+$B$^+$
RT1679	DH5α/pVJ70 *pduF′ABCDE* ORF1′ (Amp^r)
S. typhimurium	
RT786	*metE205 ara-9 pduF89*::Tn*10*dKan
TR6579	*metA22 metE551 trpD2 ilv-452 hsdLT6 hsdSA29* HsdB$^-$ *strA120* GalE$^-$ Leu$^-$ Pro$^-$
TT10324	*metE205 ara-9 cob-21*::MudA (Amp^r)
TT11855	*metE205 ara-9* DEL299 (His$^-$ Pdu$^-$ Cob$^-$)
TT17951	*srl-203*::Tn*10*dCam *zec-3976*::Tn*10*dTet *pduG273*
TT17950	*srl-203*::Tn*10*dCam *zec-3976*::Tn*10*dTet *pduD272*
TT17958	*srl-203*::Tn*10*dCam *zec-3976*::Tn*10*dTet *pduD283*
TT17998	*srl-203*::Tn*10*dCam *zec-3976*::Tn*10*dTet *pduC322*
TT18011	*srl-203*::Tn*10*dCam *pduF-354*::Tn*10*dTet *pduC335*
TT18631	*metE205 ara-9* DEL299/pXY6 *pduC*
TT18632	*metE205 ara-9* DEL299/pXY7 *pduC*
TT18633	*metE205 ara-9* DEL299/pXY8 *pduCD*
TT18634	*metE205 ara-9* DEL299/pXY9 *pduCD*
TT18635	*metE205 ara-9* DEL299/pXY10 *pduCD*
TT18636	*metE205 ara-9* DEL299/pXY18 *pduCDE*
TT18637	*metE205 ara-9* DEL299/pXY19 *pduCDE*
TT18638	*metE205 ara-9* DEL299/pXY39 *pduCDE*

(31). Ampicillin was used at 100 μg/ml unless indicated otherwise. Kanamycin was used at 50 μg/ml, IPTG was used at 1 mM, and X-Gal was used at 20 mg/liter. MacConkey-propanediol-B_{12} indicator plates were composed of MacConkey agar base (Difco, Detroit, Mich.) supplemented with 1% propanediol and 200 ng of vitamin B_{12} per ml. On these indicator plates, Pdu^- strains are white but Pdu^+ strains are bright red due to acid produced from propanediol catabolism.

Molecular methods. Agarose gel electrophoresis was performed as described previously (28). Gel purification of DNA involved eluting DNA bands from agarose slices by a "freeze squeeze" procedure. For ligation of DNA fragments, T4 DNA ligase was used as specified by the manufacturer. Electroporation of low-ionic-strength cell suspensions was done with Gene Pulser (Bio-Rad, Richmond, Calif.) as specified by the manufacturer and used at the following settings: capacitance, 25 μF; capacitance extender, 250 μF; pulse controller, 200 Ω; voltage, 2.5 kV (19). Putative transformants were purified by single-colony isolation. Plasmid DNA was isolated by the TELT miniprep procedure (3). Restriction digestions were carried out by standard methods (28).

P22 transduction. Transductional crosses were performed as described previously (17) with P22 HT105/1 *int*-210, a mutant phage that has high transducing ability (37). For the preparation of P22 transducing lysates from strains with *galE* mutations, overnight cultures were grown on LB medium supplemented with 0.2% glucose and 0.02% galactose.

Cloning of the *pdu* operon fragment that includes the propanediol dehydratase genes. For use as a cloning marker, a Tn*10*dKan insertion linked to the *pdu* operon was isolated. *S. typhimurium* TT10324 (*cob*-21::MudA Amp^r) was transduced with a pool of random chromosomal Tn*10*dKan insertions (46) and the Kan^r transductants were screened for simultaneous loss of the Amp^r phenotype. One *S. typhimurium* strain (RT786) carrying a Tn*10*dKan insertion linked to the *pdu* operon was saved. To obtain *pdu* DNA, the kanamycin resistance determinant of RT786 was cloned. Chromosomal DNA was isolated, digested with *Sau*3AI, and dephosphorylated with calf intestinal alkaline phosphatase by standard molecular methods (3). Restriction fragments in the 30- to 50-kbp size range were recovered following equilibrium centrifugation in a cesium chloride density gradient and ligated into the *Bam*HI site of the cosmid vector cosKT1 (40). The ligation products were packaged into bacteriophage lambda heads in an in vitro packaging reaction (Packagene lambda DNA packaging system; Promega Corp., Madison, Wis.). *Escherichia coli* DH5α was infected with the lambda phage particles, and cosmid-bearing colonies were selected on LB agar containing 75 μg of ampicillin per ml. The Amp^r colonies were screened for kanamycin resistance and for propanediol catabolism by using MacConkey-propanediol-B_{12} indicator plates. *E. coli* strains do not degrade propanediol if the plasmid is not present. Selected colonies were isolated in pure culture, and crude cell extracts were prepared and assayed for propanediol dehydratase activity as previously described (22).

One cosmid carried a 39-kbp insert that conferred both a Kan^r phenotype and propanediol dehydratase activity to *E. coli*. Cosmid DNA was isolated by the alkali lysis method (3), digested initially with *Apa*I, and then partially digested with *Eco*RI. The resulting restriction fragments were cloned into the phagemid vector Bluescript II KS(+/−) (Stratagene Cloning Systems). One phagemid contained a 20.8-kbp *Eco*RI insert, which conferred kanamycin resistance and propanediol dehydratase activity. A 6.4-kbp *Hin*dIII fragment that expresses propanediol dehydratase was isolated and subcloned into Bluescript II KS(+/−). The recombinant phagemid was designated pVJ70 (Amp^r), and the *E. coli* DH5α host strain carrying phagemid pVJ70 was designated RT1679.

Subcloning of the pVJ70 insert. Plasmids pTA726, pTA728, pTA734, pTA731, and pTA729 were constructed by ligating restriction fragments of plasmid pVJ70 into the vector pGEM3Z (Promega). Figure 1 shows these subclones. The general subcloning procedure was to digest plasmid pVJ70 with the desired restriction enzyme(s) and to purify the DNA fragment of interest by gel electrophoresis. Isolated DNA fragments were used in ligation reactions with pGEM3Z which had been previously cut with the appropriate restriction enzymes and gel purified. Ligation reaction mixtures were used as a source of plasmid DNA for transformation of *E. coli* JM107 by electroporation. Transformed cells were plated onto LB medium containing 100 μg of ampicillin per ml, 1 mM IPTG, and 20 mg of X-Gal per ml. On this medium, strains carrying pGEM3Z with an insertion in its polylinker form white colonies but strains carrying plasmids without inserts form blue colonies. To identify plasmids with inserts of the expected molecular weights, plasmid DNA purified from transformed cells was analyzed by restriction digestion and agarose gel electrophoresis (28). Selected plasmids were used to determine the DNA sequence of the pVJ70 insert.

DNA sequencing and analysis. Plasmids pVJ70, pTA726, pTA728, pTA734, pTA731, and pTA729 (Fig. 1) were purified on Qiagen tip 100 columns (Qiagen Inc., Chatsworth, Calif.) and denatured with alkali. DNA sequencing was performed with Sequenase version 2.0 (United States Biochemical, Cleveland, Ohio) as specified by the manufacturer. Sequence compressions were resolved by the Sequenase version 2.0 dITP method. Sequence analysis and comparisons were performed with GeneMark software (10), the Genetics Computer Group (GCG) program package (18), and Blast software (2).

Construction of minimal clones of *pdu* genes. Minimal *pduC*, *pduCD*, or *pduCDE* clones were constructed so that their expression could be induced with IPTG. This was done by cloning PCR products into the expression vector p*lacI*^{q}PO-*Bgl*II. This vector contains a wild-type *lac* promoter and a Shine-Dalgarno sequence just upstream of a *Bgl*II site. If DNA cloned into the *Bgl*II site has an AUG codon adjacent to the *Bgl*II site, it will be expressed via the *lac* promoter. Since this vector also expresses the $LacI^q$ protein, transcription of the cloned DNA is inducible by IPTG. The *pdu* DNA used for making minimal clones was obtained by PCR amplification with plasmid pVJ70 as the template and the following primers: for *pduC* amplification, GGAATTCAGATCTATG AGATCGAAAAGATTTGAAGCACT and GGAATTCAAGCTTCTTAATC AATCTCGTTGGGATCAAGA; for *pduCD* amplification, GGAATTCAGAT CTATGAGATCGAAAAGATTTGAAGCACT and GGAATTCAAGCTTTA TTCATGGAGTTATCCTTTATCAAAGC; for *pduCDE* amplification, GGAA TTCAGATCTATGAGATCGAAAAGATTTGAAGCACT and AAGGATCC AAGCTTTCGCATACGAAATCCTTAATCGTC. Because of its high fidelity, the *pfu* DNA polymerase (Stratagene, La Jolla, Calif.) was used for the PCR amplification. This polymerase was used as specified by the manufacturer, with the modification that 10% dimethylsulfoxide and 150 μg of additional bovine serum albumin per ml were included in each reaction mixture. The amplified DNA was purified with Wizard PCR Preps (Promega) and digested with *Bgl*II and *Hin*dIII; this cleaved the restriction sites designed into the PCR primer sequences. The digestion products were again purified with Wizard PCR Preps and then ligated into purified p*lacI*^{q}PO-*Bgl*II vector that had also been cut with *Bgl*II and *Hin*dIII. A portion of each ligation reaction mixture was used to transform *S. typhimurium* TR6579 by electroporation. Transformants were obtained by selecting for ampicillin resistance on LB medium. Plasmid DNA was isolated from selected colonies and checked for inserts of the appropriate size. Plasmids with inserts of the expected size were transferred to strain TT11855 by P22 transduction. The resulting strains (TT18631 to TT18638) were used in complementation and expression studies.

Complementation tests. P22 transduction was used to transfer *pduC* or *pduCD* clones from strains TT18631 to TT18635 into several *S. typhimurium* strains that each carried a propanediol dehydratase point mutation (TT17950, TT17958, TT17998, and TT18011). The resulting merodiploids were qualitatively tested for propanediol dehydratase activity. This was done by plating transduction mixtures onto MacConkey-propanediol-B_{12} indicator plates supplemented with ampicillin and IPTG. The plates were incubated overnight at 37°C. The presence of red colonies indicated production of propanediol dehydratase. If all the transductant colonies resulting from a given cross were red on the supplemented MacConkey indicator medium, this was interpreted as complementation of the recipient propanediol dehydratase mutation by the donated clone.

Determination of propanediol dehydratase expression from the *pduC*, *pduCD*, and *pduCDE* clones. Cell extracts used to determine propanediol dehydratase expression were prepared by growing an overnight culture of each strain in 2 ml of LB medium plus ampicillin at 37°C. This culture (0.1 ml) was used to inoculate 2 ml of NCE medium supplemented with 1% succinate, 0.1 mM histidine, 0.3 mM methionine, 1 mM $MgSO_4$, and 1 mM IPTG. These cultures were incubated at 37°C overnight, and 0.5 ml was used to inoculate 25 ml of similar minimal medium. The 25-ml cultures were grown at 37°C until their absorbance at 650 nm was 0.6 to 0.8. The cells were then pelleted by centrifugation and resuspended in 2 ml of 70 mM potassium phosphate (pH 8.0). The centrifugation and resuspension steps were repeated. The cell suspensions were lysed by sonication with a no. 450 sonifier (Branson Scientific, Danbury, Conn.) under the following conditions: microtip, output control set at 5, 50% duty cycle, 75 pulses, samples on ice. Cell debris were pelleted by centrifugation for 10 min at 4°C in Fisher 235C microcentrifuge. The supernatants were the cell extracts used in propanediol dehydratase assays. These extracts were stored at −80°C and used within 2 days.

Propanediol dehydratase activity was quantified by measuring the formation of propionaldehyde. This was done by monitoring the conversion of NADH to NAD in a coupled NADH-dependent alcohol dehydrogenase reaction that reduces propionaldehyde to propanol (4). The 3-ml reaction mixtures included the following components: cell extract, 40 μl; (*S*)-1,2-propanediol, 0.1 mM; excess alcohol dehydrogenase, 18 U; HEPES buffer (pH 7.5), 100 mM; Ado-CBL, 12.7 μM. The reactions were initiated (with the room lights off) by the addition of 30 μl of a 2-mg/ml aqueous solution of Ado-CBL. The reaction temperature was 37°C, and the absorbance at 340 nm was monitored with a Cary 219 spectrophotometer. For quantitation, an ε_{340} for NADH of 6,220 M^{-1} cm^{-1} was used. One unit of propanediol dehydratase activity was defined as the amount of propionaldehyde (in micromoles) formed per minute per milligram of protein. Protein was determined by the method of Bradford with Coomassie protein assay reagent (Pierce, Rockford, Ill.) as specified by the manufacturer (11).

Accession number. The sequence reported here has been submitted to GenBank and assigned accession no. AF026270.

RESULTS

Cloning of the propanediol dehydratase genes. A mutant of *S. typhimurium* that carried a Tn*10*dKan insertion linked to the *pdu* operon was isolated, and subsequently a cosmid clone carrying this marker was identified. Of 780 Amp^r (cosKT1-bearing) colonies, 31 were Kan^r. The Kan^r strains were screened for Pdu phenotypes. No strain displayed a B_{12}-dependent Pdu^+ phenotype on MacConkey-propanediol plates,

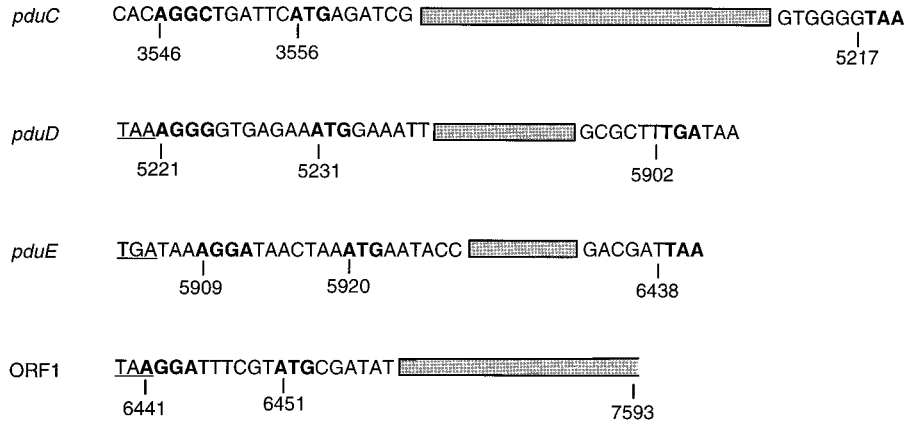

FIG. 2. Sequence features of *pduCDE* genes and ORF1. See GenBank sequence AF026270. Ribosome binding sites, start codons, and stop codons are shown in boldface type. The stop codon of the previous gene is underlined.

indicating that none of the cosmids had an insert encoding the entire *pdu* operon. However, 1 of the 31 strains produced propanediol dehydratase activity in an in vitro enzyme assay of crude cell extracts (data not shown). This cosmid contained a 39-kbp insert. A 6.4-kbp *Hin*dIII fragment from the cosmid was subcloned to form plasmid pVJ70, which also expressed propanediol dehydratase activity (data not shown).

DNA sequence of the clone that expresses propanediol dehydratase. The DNA sequence of the pVJ70 insert was assembled by combining new and previous data (12). This completed the DNA sequence of the first five genes of the *pdu* operon (*pduABCDE*) and part of a sixth, ORF1 (Fig. 1). The DNA sequences of the subcloned inserts in plasmids pTA728, pTA734, pTA731, and pTA729 were determined on both strands. The juxtaposition of these inserts was established by sequencing appropriate regions of pTA726. The DNA sequence of additional segments of the pVJ70 clone was determined on both strands until overlap with vector or a previously determined sequence was apparent. Thus, data were acquired that allowed the complete DNA sequence the pVJ70 insert to be assembled. The pVJ70 insert included 3,861 bp of new *pdu* sequence, 2,083 bp of previously determined *pdu* sequence, and 397 bp of sequence derived from the Tn*10*dKan element used as a selectable marker for cloning. The Tn*10*dKan insertion had disrupted the *pduF* gene and was oriented with its right end downstream of the transcriptional start of the *pduF* gene. Like other *pduF* insertions, the Tn*10* insertion did not confer any obvious mutant phenotype (12). The PduF protein facilitates diffusion of propanediol (12, 13) but is not necessary for formation of red colonies on MacConkey-propanediol-B_{12} indicator plates.

DNA sequence analyses. Three new genes, *pduCDE*, and one partial gene, ORF1, were identified by DNA sequence analyses (Fig. 1). The determined *pduC* sequence completed a previously reported partial ORF (12). Ribosome binding sites were determined by visual inspection (Fig. 2). For the *pduDE* genes and ORF1, the ribosome binding site was adjacent to or overlapped the stop site of the previous gene. The codon adaptation indexes of the *pduCDE* genes and ORF1 indicated nonrandom codon usage that is typical of expressed *S. typhimurium* sequences (Table 2). Also, the G+C contents of each codon position showed biases indicative of expressed sequences (Table 2).

Further analyses of the *pduCDE* genes and ORF1 supported previous evidence (25, 36) that *Salmonella* acquired the *pdu* and *cob* operons by a single horizontal gene transfer (Table 2). The G+C content of the coding regions of the *pduCDE* genes and ORF1 averages about 57%, which is higher than the 54% average G+C content typical of *S. typhimurium* genes. The G+C contents of the first, second, and third codon positions are also atypical of the *S. typhimurium* coding sequencing. However, the G+C composition of the *pduCDE* genes and ORF1 is similar to that of the adjacent *cob* operon, for which there is substantial evidence of horizontal transfer (24, 25).

By using the GCG tfasta and fasta programs (18) and the Blastn, Blastp, and Blastx software (2), the amino acid and nucleotide sequences of the *pduCDE* coding regions and ORF1 were compared to sequences in GenBank (Table 3). In terms of nucleotide sequence, the *pduCDE* genes from *S. typhimurium* are 93% identical to the *pddABC* genes from *K. oxytoca*, 68% identical to the *dhaBCE* genes from *C. freundii*, and 67% identical to the *gldABC* genes from *K. pneumoniae*. In amino acid sequence, the PduCDE enzyme is 98, 65, and 65% identical to the PddABC, DhaBCE, and GldABC enzymes, respectively. The DhaBCE and the GldABC proteins are Ado-CBL-dependent glycerol dehydratases (38, 41). The PddABC enzyme is an Ado-CBL-dependent propanediol dehydratase (42). The identity between the *S. typhimurium* PduCDE enzyme and the *K. oxytoca* PddABC enzyme is much higher than would be expected based on the relationship of these organ-

TABLE 2. Codon usage bias and G+C content of the *S. typhimurium pduCDE* and ORF1 coding sequences

ORF	ORF length (bp)	CAI[a]	%G+C by position[b]:			% G+C[c]
			1st	2nd	3rd	
pduC	1,662	0.465	61	41	69	57
pduD	672	0.377	63	42	66	57
pduE	519	0.433	63	43	63	56
ORF1	1,143	0.323	62	43	69	58
pduCDE ORF1			61	42	68	57
cob operon			63	44	61	56
Typical gene of *S. typhimurium*		0.2–0.8	58	44	58	54

[a] The codon adaptation index was determined as described previously (39).
[b] Percent G+C content of the first, second, or third codon position of the indicated coding sequence.
[c] Percent G+C content of the indicated coding sequence.

TABLE 3. Sequences homologous to the *S. typhimurium pduCDE* genes and *pdu* ORF1

Salmonella gene[a]	Homolog[b]		Gene length (bp) of:		Protein mol mass (Da) of:		% Identity[c] for:	
	Gene[a]	Organism	*Salmonella* gene	Homolog	*Salmonella* protein	Homolog	Nucleotides	Amino acids
pduC	*pddA*	*K. oxytoca*	1,662	1,662	60,307	60,348	93.8	99.6
pduD	*pddB*	*K. oxytoca*	672	672	24,157	24,113	91.8	96.9
pduE	*pddC*	*K. oxytoca*	519	519	19,131	19,173	93.0	97.1
pduC	*dhaB*	*C. freundii*	1,662	1,668	60,307	60,433	69.4	70.9
pduD	*dhaC*	*C. freundii*	672	585	24,157	21,487	61.8	58.6
pduE	*dhaE*	*C. freundii*	519	429	19,131	16,121	57.4	51.7
pduC	*gldA*	*K. pneumoniae*	1,662	1,668	60,307	60,621	71.1	70.5
pduD	*gldB*	*K. pneumoniae*	672	585	24,157	21,310	61.3	58.9
pduE	*gldC*	*K. pneumoniae*	519	426	19,131	16,094	56.2	53.1
ORF1	ORFZ	*C. freundii*	1,143	1,868		65,340	59.6	54.8

[a] The *pduCDE* genes of *S. typhimurium* and the *pddABC* genes of *K. oxytoca* encode Ado-CBL-dependent propanediol dehydratases (42; see the text). The *dhaBCE* genes of *C. freundii* and the *gldABC* genes of *K. pneumoniae* encode Ado-CBL-dependent glycerol dehydratases (38, 41).
[b] To identify homologs, the coding sequences of the *pduCDE* genes were compared to sequences in GenBank by using GCG fasta and tfasta (18) and Blastn, Blastp, and Blastx (2).
[c] The percent identity was determined by using the GCG GAP program.

isms. This finding is discussed below. Comparison of the *pdu* ORF1 to GenBank sequences identified one related sequence. ORF1 is 60% identical to the first 1,125 residues of ORFZ from *C. freundii*. The inferred proteins encoded by ORF1 and the 5′ end of ORFZ are 55% identical. However, the function of ORFZ is unknown (38). The proteins encoded by the *pduCDE* genes and ORF1 were also searched for motifs related to those in the Prosite dictionary by using GCG software; however, no matches were found.

Complementation analysis and independent expression of *pduC*, *pduCD*, and *pduCDE* clones. Eight clones (two of the *pduC* gene, three of the *pduCD* genes, and three of the *pduCDE* genes) were tested for their ability to complement selected propanediol dehydratase point mutations (Table 4) and to express propanediol dehydratase (Table 5). The propanediol dehydratase point mutations used were previously shown by complementation analyses to be in the *pduC* or *pduD* genes (45).

Minimal clones of the *pduC* gene complemented *pduC* point mutations but not *pduD* point mutations (Table 4). This indicated that the PduC protein was functionally expressed from the plasmid and is essential for propanediol dehydratase activity. It also shows that the *pduC* complementation group corresponds to the *pduC* gene identified by DNA sequence analysis. The finding that the *pduC* clone failed to complement some propanediol dehydratase mutations indicates that proteins in addition to PduC are required for propanediol dehydratase activity. Strains containing plasmids with *pduC* inserts that are functional in complementation tests did not produce enzymatic activity when placed in a strain with a deletion of the entire *pdu* operon (Table 5). This further indicated that polypeptides in addition to PduC were required for propanediol dehydratase activity.

Plasmids containing *pduCD* clones restored propanediol dehydratase activity to all propanediol dehydratase-deficient point mutants tested, including strains carrying mutations in either the *pduC* or *pduD* complementation group. This indicates that these clones express functional PduC and PduD proteins and that these peptides are both required for propanediol dehydratase activity. These results also show that the *pduC* and *pduD* complementation groups identified genetically

TABLE 4. Complementation of chromosomal propanediol dehydratase mutations by minimal clones

Strain	Propanediol dehydratase point mutation[d]	Complementation[a] for plasmid[b] (insert[c])			
		Vector (none)	pXY6,7 (*pduC*)	pXY8,9,10 (*pduCD*)	pXY18,19,39 (*pduCD*)
TT17998	*pduC322*	−	+	+	+
TT18011	*pduC335*	−	+	+	+
TT17950	*pduD272*	−	−	+	+
TT17958	*pduD283*	−	−	+	+

[a] Complementation was indicated by restoration of propanediol dehydratase activity to mutant strains of *S. typhimurium*. This was detected as acid production on MacConkey-propanediol-vitamin B_{12} indicator medium supplemented with 1 mM IPTG and 100 μg of ampicillin per ml. As negative controls, strains TT18631 to TT18638 were shown not to produce acid on MacConkey-propanediol-vitamin B_{12} indicator plates.
[b] All plasmids were derivatives of the vector p*lacI*^{q}PO-*Bgl*II.
[c] Inserts were the coding sequences of the indicated gene(s) (they are diagrammed at the bottom of Fig. 1). The *lac* promoter of the plasmid provided for expression of the cloned sequences.
[d] The propanediol dehydratase point mutations used were previously identified by complementation tests (45).

TABLE 5. Expression of propanediol dehydratase activity by minimal clones

Strain[b]	Plasmid[c]	Insert[d]	Dehydratase activity[a] with IPTG:	
			Absent	Present[e]
TT18631	pXY6	*pduC*	<0.1	<0.1
TT18632	pXY7	*pduC*	<0.1	<0.1
TT18633	pXY8	*pduCD*	<0.1	<0.1
TT18634	pXY9	*pduCD*	<0.1	<0.1
TT18635	pXY10	*pduCD*	<0.1	<0.1
TT18636	pXY18	*pduCDE*	<0.1	8.0
TT18637	pXY19	*pduCDE*	<0.1	2.0
TT18638	pXY39	*pduCDE*	<0.1	12.3

[a] Extracts, prepared from cells grown on minimal medium in the presence or absence of IPTG, were assayed for propanediol dehydratase activity.
[b] Strains TT18631 to TT18638 each contain a large chromosomal deletion that removes the entire *pdu* operon; hence, propanediol dehydratase activity measured in these strains is attributable to plasmid expression.
[c] Plasmids are derivatives of the expression vector p*lacI*^{q}PO-*Bgl*II carrying the indicated insert.
[d] Inserts were the coding sequences of the indicated genes.
[e] IPTG was necessary for the expression of propanediol dehydratase activity because inserts were cloned such that their transcription was under control of the *lac* promoter and the *lacI*^{q} gene product.

correspond to the *pduCD* genes identified by sequence analysis. Although *pduCD* clones complemented all tested mutations in the two known propanediol dehydratase complementation groups, they did not allow the expression of propanediol dehydratase activity in a strain with a deletion of the entire *pdu* operon (Table 5). This indicates that proteins in addition to the PduCD proteins (and not identified by complementation analysis) are required for propanediol dehydratase function.

Plasmids containing a precise *pduCDE* insert expressed high levels of propanediol dehydratase activity in a *pdu* deletion strain, and they did so only in the presence of IPTG (Table 5). As expected, *pduCDE* clones also complemented all propanediol dehydratase point mutations tested (Table 4). As negative controls for the complementation tests, strains TT18631 to TT18638 were shown not to produce acid on MacConkey-propanediol-B12 indicator plates. Cumulatively, the results of the complementation and expression studies demonstrate that the PduCDE proteins are necessary and sufficient for propanediol dehydratase activity in *S. typhimurium.*

DISCUSSION

To better understand vitamin B_{12} physiology, we have further characterized the *pdu* operon of *S. typhimurium*. The *pdu* operon encodes enzymes of the propanediol catabolic pathway, including an Ado-CBL-dependent propanediol dehydratase (22, 32, 43, 45). Here we showed that three genes (*pduCDE*) are necessary and sufficient for the expression of propanediol dehydratase activity. Prior genetic studies showed that the *pdu* operon also encodes a CoA-dependent propionaldehyde dehydrogenase, but the relevant genes were not identified (45). In addition to the dehydratase and the dehydrogenase mentioned, the *pdu* operon is thought to encode phosphotransacylase, propionate kinase, and propanol dehydrogenase (32, 43), but this has not yet been established by genetic tests.

Studies of the *pdu* operon have shown that it contains 8 to 13 genes in addition to those that catalyze steps of the propanediol catabolic pathway (8, 24). Here we identified a partial *pdu* ORF, ORF1, that is homologous to ORFZ of *C. freundii*. ORFZ is located in the *dha* regulon of *C. freundii* together with genes for the dihydroxyacetone pathway of glycerol fermentation, including those that encode an Ado-CBL-dependent glycerol dehydratase (15, 38). Apparently, all the *dha* genes that encode pathway enzymes have been identified (14, 16, 38). This suggests that ORF1 encodes a function, other than a pathway enzyme, that is common to the Ado-CBL-dependent catabolism of propanediol and glycerol. ORF1 might be involved in the conversion of CN-CBL to Ado-CBL. Genetic tests have suggested that at least one *pdu* gene, *pduG*, is involved in this process (45).

There are a number of other functions and possible functions for *pdu* genes. Some are needed for the normal functioning of the polyhedral bodies that *S. typhimurium* forms when catabolizing propanediol (8, 45). Some might function in the Ado-CBL-dependent anaerobic respiration of propanediol with tetrathionate as the terminal electron acceptor. In addition, the work reported here suggests other possibilities.

Here we showed that the propanediol dehydratase enzymes of *S. typhimurium* and *K. oxytoca* are nearly identical (Table 3). This suggests that biochemical studies of the *K. oxytoca* enzyme will apply to the *S. typhimurium* enzyme. The propanediol dehydratase of *K. oxytoca* may be a membrane-associated enzyme (29, 30). The enzyme is subject to inactivation by glycerol but can be reactivated in situ by a glycerol-inducible system (20). The minimal *Klebsiella* enzyme, *pddABC*, is somewhat heat sensitive for unknown reasons (42). Thus, Ado-CBL-dependent propanediol dehydratase from *K. oxytoca* (and *S. typhimurium*) may require additional proteins that improve heat stability or membrane interaction and/or that play a role in enzyme reactivation. Such proteins may be encoded by the *pdu* operon.

Previous work, and the work reported here completes about one-half of the DNA sequence of the *pdu* operon. The DNA sequences of the first five genes (*pduABCDE*) and part of a sixth gene (ORF1) are now known (12, 36; see above). The first six genes of the *pdu* operon have an atypical G+C content. This finding further supports prior investigations indicating that the *cob* and *pdu* operons of *S. typhimurium* were acquired by a recent introgression of foreign DNA (25). Unexpectedly, the *pduCDE* genes of *S. typhimurium* and the *pddABC* genes of *K. oxytoca* were 94, 92, and 93% identical in nucleotide sequence, respectively (Table 3). This level of divergence is of the order expected for intraspecific variance: 0.4 to 8.0% (39). Typically, the identity among homologous genes from *Klebsiella* and *Salmonella* is about 80% (8). This suggests that the *Klebsiella pdd* genes may also have been acquired by horizontal transfer, and this inference is consistent with the proposal that the capacity for de novo B_{12} synthesis and propanediol degradation has been lost and regained within the enteric lineage as the species diverged (25, 35).

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