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ORIGINAL PAPER

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PduP is a coenzyme-a-acylating propionaldehyde dehydrogenase associated with the polyhedral bodies involved in B_{12}-dependent 1,2-propanediol degradation by *Salmonella enterica* serovar Typhimurium LT2

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Abstract *Salmonella enterica* forms polyhedral bodies involved in coenzyme-B_{12}-dependent 1,2-propanediol degradation. Prior studies showed that these bodies consist of a proteinaceous shell partly composed of the PduA protein, coenzyme-B_{12}-dependent diol dehydratase, and additional unidentified proteins. In this report, we show that the PduP protein is a polyhedral-body-associated CoA-acylating aldehyde dehydrogenase important for 1,2-propanediol degradation by *S. enterica*. A PCR-based method was used to construct a precise nonpolar deletion of the gene *pduP*. The resulting *pduP* deletion strain grew poorly on 1,2-propanediol minimal medium and expressed 105-fold less propionaldehyde dehydrogenase activity (0.011 μmol min^{-1} mg^{-1}) than did wild-type *S. enterica* grown under similar conditions (1.15 μmol min^{-1} mg^{-1}). An *Escherichia coli* strain was constructed for high-level production of His_8-PduP, which was purified by nickel-affinity chromatography and shown to have 15.2 μmol min^{-1} mg^{-1} propionaldehyde dehydrogenase activity. Analysis of assay mixtures by reverse-phase HPLC and mass spectrometry established that propionyl-CoA was the product of the PduP reaction. For subcellular localization, purified His_8-PduP was used as antigen for the preparation of polyclonal antiserum. The antiserum obtained was shown to have high specificity for the PduP protein and was used in immunogold electron microscopy studies, which indicated that PduP was associated with the polyhedral bodies involved in 1,2-propanediol degradation. Further evidence for the localization of the PduP enzyme was obtained by showing that propionaldehyde dehydrogenase activity co-purified with the polyhedral bodies. The fact that both Ado-B_{12}-dependent diol dehydratase and propionaldehyde dehydrogenase are associated with the polyhedral bodies is consistent with the proposal that these structures function to minimize propionaldehyde toxicity during the growth of *S. enterica* on 1,2-propanediol.

Keywords 1,2-Propanediol · B12 · Polyhedral organelles · Aldehyde dehydrogenase · Carboxysomes

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Introduction

Virtually all *Salmonella* degrade 1,2-propanediol in a coenzyme-B_{12}-dependent manner and this ability (which is absent from *Escherichia coli*) is thought to be an important aspect of the *Salmonella*-specific lifestyle (Roth et al. 1996; Price-Carter et al. 2001). The degradation of 1,2-propanediol may play a role in the interaction of *Salmonella* with its host organisms. In *Salmonella enterica*, in vivo expression technology (IVET) has indicated that 1,2-propanediol utilization (*pdu*) genes may be important for growth in host tissues, and competitive index studies with mice have shown that *pdu* mutations confer a virulence defect (Conner et al. 1998; Heithoff et al. 1999). 1,2-Propanediol is likely to be abundant in anoxic environments, such as the large intestine, since it is produced by the anaerobic degradation of the common plant sugars rhamnose and fucose. In addition, 1,2-propanediol degradation by *S. enterica* provides an important model system for understanding coenzyme-B_{12}-dependent processes, some of which are important in human physiology, industry, and the environment (Roth et al. 1996).

On the basis of biochemical studies, a pathway for coenzyme-B_{12}-dependent 1,2-propanediol degradation by *S. enterica* was proposed (Fig. 1). This pathway begins with the conversion of 1,2-propanediol to propionaldehyde, a reaction that is catalyzed by coenzyme-B_{12}-dependent diol dehydratase (Toraya et al. 1979; Obradors et al. 1988). The aldehyde is then disproportionated to propanol and propionic acid by a reaction series thought to involve propanol dehydrogenase, coenzyme-A (CoA)-dependent propionaldehyde dehydrogenase, phosphotransacylase, and propionate kinase (Toraya et al. 1979; Obradors et al.

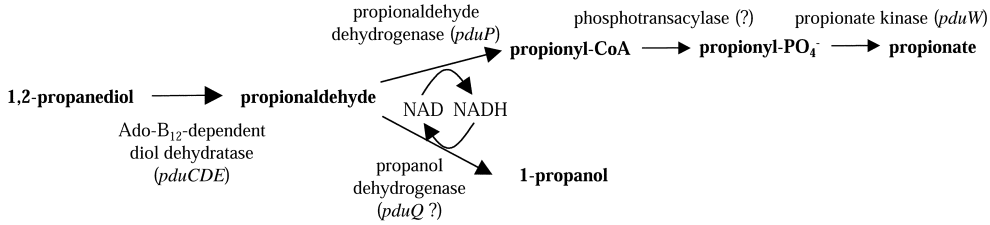

Fig. 1 Proposed pathway of Ado-B_{12}-dependent 1,2-propanediol degradation

1988; Palacios et al. 2003). This pathway generates one ATP, an electron sink, and a 3-carbon intermediate (propionyl-CoA), which can feed into central metabolism via the methyl-citrate pathway (Horswill and Escalante-Semerena 1997). In *S. enterica*, the degradation of 1,2-propanediol occurs aerobically, or anaerobically when tetrathionate is supplied as an electron acceptor (Price-Carter et al. 2001).

The genes specifically required for growth of *S. enterica* on 1,2-propanediol are organized as a single contiguous cluster named the propanediol utilization (*pdu*) locus. Determination of the DNA sequence of this locus showed that 1,2-propanediol degradation was much more complex than prior biochemical studies had suggested. DNA sequence analyses indicated the *pdu* locus included 23 genes (Bobik et al. 1997, 1999): six *pdu* genes are thought to encode enzymes needed for the 1,2-propanediol degradative pathway (Bobik et al. 1997); two are involved in transport and regulation (Bobik et al. 1992; Chen et al. 1994); two are probably involved in diol dehydratase reactivation (Bobik et al. 1999); one is needed for the conversion of vitamin B_{12} (CN-B_{12}) to coenzyme B_{12} (Johnson et al. 2001); five are of unknown function; and seven share similarity to genes involved in the formation of carboxysomes, polyhedral bodies found in certain cyanobacteria and some chemoautotrophs (Shively and English 1991; Shively et al. 1998).

The finding that the *pdu* locus included a number of genes similar to those involved in carboxysome formation suggested that a related polyhedral body might be involved in B_{12}-dependent 1,2-propanediol degradation. Indeed, *S. enterica* was recently shown to form polyhedral bodies (also called polyhedral organelles) during growth on 1,2-propanediol (Bobik et al. 1999; Havemann et al. 2002). In general appearance, the *pdu* polyhedra are similar to carboxysomes. They are 100–150 nm in cross-section and composed of a proteinaceous interior covered by a 3- to 4-nm protein shell. However, carboxysomes and the *pdu* polyhedra differ in several ways. Carboxysomes, which contain most of the cell's ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO), function to improve autotrophic growth at low CO_2 concentrations and are thought to do so by concentrating CO_2 (Shively et al. 2001; Price et al. 2002). By contrast, the *pdu* polyhedra of *S. enterica* do not contain RuBisCO. They consist of a protein shell composed in part of the PduA protein (Havemann et al. 2002) and are associated with B_{12}-dependent diol dehydratase and other unidentified proteins (Bobik et al. 1999). The function of the *S. enterica* polyhedral bodies is uncertain. Work published to date suggests that they may serve to minimize aldehyde toxicity by sequestration and channeling of propionaldehyde and/or by moderating the rate of aldehyde formation through control of diol dehydratase activity (Chen et al. 1994; Rondon et al. 1995; Stojiljkovic et al. 1995; Bobik et al. 1999; Havemann et al. 2002; Havemann and Bobik 2003).

If the *pdu* polyhedra do indeed function to minimize aldehyde toxicity, it might be expected that aldehyde-degrading enzymes are also associated with these structures. In this report, we show that *pduP* encodes a polyhedral-body-associated CoA-acylating propionaldehyde dehydrogenase important for 1,2-propanediol degradation.

Materials and methods

Bacterial strains, media, and growth conditions

The bacterial strains used in this study are listed in Table 1. The rich medium used was Luria-Bertani/Lennox (LB) medium (Difco, Detroit, Mich., USA) (Miller 1972). The minimal medium used was no-carbon-E (NCE) medium (Vogel and Bonner 1956; Berkowitz et al. 1968) supplemented with 53 mM propanediol, 1 mM $MgSO_4$, 148 nM CN-B_{12}, and 0.3 mM each valine, isoleucine, leucine, and threonine. In addition, for strains carrying pLAC22 media were also supplemented with 1 mM IPTG, and 100 µg Amp/ml. Cultures were incubated at 37 °C with shaking at 250 rpm as previously described (Leal et al. 2003), and cell growth was determined by measuring the optical density of cultures at 600 nm.

General molecular methods

Agarose gel electrophoresis was done as described previously (Sambrook et al. 1989). Plasmid DNA was purified by the alkaline lysis procedure (Sambrook et al. 1989) or by using Qiagen products (Qiagen, Chatsworth, Calif., USA) according to the manufacturer's instructions. Following restriction or PCR amplification, DNA was purified using Qiagen PCR purification or gel extraction kits. Restriction enzymes were used according to standard protocols (Sambrook et al. 1989). DNA fragments were ligated using T4 DNA ligase according to the manufacturer's directions. Electroporation was carried out as previously described (Bobik et al. 1999).

General protein methods

SDS-PAGE was carried out using Bio-Rad Redigels and Bio-Rad Mini-Protean II electrophoresis cells according to the manufacturer's instructions. Following gel electrophoresis, proteins were stained with Coomassie Brilliant Blue R-250. The protein concentration of solutions was determined using Bio-Rad protein assay reagent (Bio-Rad).

Table 1 Bacterial strains

Species	Strain	Genotype
Escherichia coli		
	BL21 (DE3) RIL	(*E. coli* B) F$^-$*ompT hsdS* ($r_B^-m_B^-$) *dcm*$^+$ Tet^r *gal* λ (DE3) *endA* Hte (*argU ileY leuW* Cam^r)
	BE237	BL21 (DE3) RIL/pET-41a (T7 expression vector without insert, Kan^r)
	BE273	BL21 (DE3) RIL/pNL69 (T7 expression vector with insert encoding His8-PduP)
	S17.1*λpir*	*recA*(RP4–2-Tc::Mu) *λpir*
	BE47	*thr-480*::Tn*10*dCam
Salmonella enterica serovar Typhimurium LT2		
	TR6579	*metA22 metE551 trpD2 ilv-452 hsdLt6 hsdSA29* HsdB$^-$ *strA120* GalE$^-$Leu$^-$Pro$^-$
	BE191	*ΔpduP*659
	BE268	LT2/ pNL9 (pLAC22-*pduP*, Ap^r)
	BE269	*ΔpduP*659/ pLAC22 (vector without insert, Ap^r)
	BE270	*ΔpduP*659/pNL9 (pLAC22-*pduP*, Ap^r)

P22 transduction

Transductional crosses were done as described using P22 HT105/1 *int*-210 (Davis et al. 1980), a mutant phage that has high transducing ability (Schmieger 1971). For the preparation of P22 transducing lysates from strains having *galE* mutations, overnight cultures were grown on LB-medium supplemented with 11 mM glucose and 11 mM galactose. Transductants were tested for phage contamination and sensitivity by streaking on green plates against P22 H5.

Cloning *pduP* into pLAC22

PCR was used to amplify *pduP* from the template pMGS2 (Havemann et al. 2002). The primers used for amplification were 5′-GGAATTCGGATCCTATGAATACTTCTGAACTCGAAAC-3′ (forward) and 5′-GGAATTCAAGCTTCAGTTAGCGAATAGAAAAGCC-3′ (reverse). These primers introduced *Bam*HI and *Hin*dIII restriction sites that were used for cloning into pLAC22 cut with *Bgl*II and *Hin*dIII (Warren et al. 2000). Cloning was carried out by ligation of the complementary cohesive ends formed by cutting with *Bam*HI and *Bgl*II since *pduP* contains an internal *Bgl*II site. Following ligation, clones were introduced into *S. enterica* strain TR6579 by electroporation and transformants were selected by plating on LB-agar supplemented with ampicillin (Amp) at 100 µg/ml. Pure cultures were prepared from selected transformants, and plasmid DNA isolated from these strains was cut with *Nru*I, *Pst*I, and *Hin*dIII. The DNA sequence of one clone that released products of the expected sizes following restriction (2,635, 3,322 and 943 bp) was determined and found to be identical to the previously published DNA sequence of *pduP* (Bobik et al. 1999). This clone (pNL9) as well as pLAC22 without insert were moved into strain BE191 by P22 transduction and the resulting strains (BE270 and BE269) were used for the complementation studies.

Construction of His_8-PduP

PCR was used to fuse an N-terminal His-tag to the PduP protein. Template pMGS2 was used with the following primers: 5′-GGGGATCCATG(CAT)$_8$ATGAATACTTCTGAACTCGAAAC-3′ (forward); and 5′-GGAATTCAAGCTTCAGTTAGCGAATAGAAAAGCC-3’ (reverse). These primers introduced *Bam*HI and *Hin*dIII restriction sites that were used for cloning into pTA925 cut with *Bgl*II and *Hin*dIII. The ligation mixture was used to transform *E. coli* strain DH5α and transformants were selected by plating on LB agar supplemented with 25 µg kanamycin/ml. Plasmid DNA isolated from selected transformants was analyzed by restriction mapping and DNA sequencing. One plasmid encoding His_8-PduP (pNL69) was introduced into *E. coli* expression strain BL21 DE3 RIL by electroporation to form expression strain BE273.

Purification of His_8-PduP under denaturing and nondenaturing conditions

For purification of PduP under denaturing conditions, *E. coli* strain BE273 was grown in 500 ml LB Kan (25 µg/ml) broth at 37 °C with shaking at 275 rpm in a 1-l baffled Erlenmeyer flask. Cells were grown to an optical density of 0.6–0.8 at 600 nm and protein production was induced by the addition of IPTG to 1 mM. Cells were incubated for an additional 2 h and harvested by centrifugation at 6,690×*g* for 10 min using a Beckman JLA-10.500 rotor. Two grams of cells (wet weight) were resuspended in 3 ml of 50 mM sodium phosphate, 300 mM NaCl, pH 7, and broken using a French pressure cell at 138,000 kPa (SLM Aminco, Urbana, Ill., USA). The protease inhibitor phenylmethylsulfonylflouride was added to the cell extract to a concentration of 100 µg/ml. To separate soluble and insoluble fractions, cell extract was centrifuged at 31,000×*g* for 30 min using a Beckman JA-20 rotor. Inclusion bodies were purified from the insoluble fraction using BPER-II according to the manufacturer’s instructions. Purified inclusion bodies were solubilized overnight in binding buffer (10 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9, and 6 M urea) and filtered using a 0.45-µm filter. Affinity purification was carried out using a 1-ml Amersham Pharmacia Hi-trap chelating (Ni^{2+}) column according the manufacturer’s instructions with the following modifications: 6 M urea was added to all buffers, and two wash steps were used, the first with 20 mM imidazole and the second with 60 mM imidazole. PduP was then eluted using 150 mM imidazole.

For purification of PduP under nondenaturing conditions, inclusion bodies (isolated as described above) were resuspended in 50 mM potassium phosphate, pH 7, 25 mM NaCl and stored at 4 °C for 24 h. Insoluble proteins were pelleted by centrifugation and PduP that remained in the supernatant was purified by nickel-affinity chromatography using Ni-NTA resin (Qiagen) according to the manufacturer’s instructions. Briefly, the column was washed with five bed volumes of binding buffer (50 mM sodium phosphate, 300 mM NaCl, pH 7) and PduP was eluted with binding buffer supplemented with 40 mM imidazole.

Propionaldehyde dehydrogenase assays

Two assays for propionaldehyde dehydrogenase activity were used. Assay one was carried our as previously described (Walter et al. 1997). Reaction mixtures contained 50 mM CHES (2-[*N*-cyclohexylamino]ethanesulfonic acid), pH 9.5, 10 mM propionaldehyde, 1 mM dithiothreitol, 75 μM NAD^+, 100 μM HS-CoA, and an appropriate amount of cell extract. Assays were incubated at 37 °C. Enzyme activity was measured by following the conversion of NAD to NADH by monitoring the absorbance of reaction mixtures at 340 nm, and the amount of NADH formed was determined using $\Delta\varepsilon_{340}$=6.22 mM^{-1} cm^{-1}. Assay two was done as described above except that dithiothreitol was omitted and the absorbance of the reaction mixtures was followed at 232 nm. An increase in absorbance at 232 nm occurs when thioester bonds are formed and $\Delta\varepsilon_{232}\cong$4.5 mM^{-1} cm^{-1} (Dawson et al. 1969).

Identification of propionyl-CoA a product of the propionaldehyde dehydrogenase reaction

Propionaldehyde dehydrogenase reactions were done as described above except that CHES buffer was replaced by 50 mM potassium phosphate, pH 7. Immediately after the reactions reached completion, they were analyzed by reverse-phase HPLC using conditions previously described (Bobik and Rasche 2003). For the purification of reaction products, HPLC solvents were buffered with 10 mM ammonium formate, pH 6.4. Selected reaction products were collected, lyophilized, resuspended in distilled water at a concentration of 4 μM. For the MALDI-TOF MS (matrix assisted laser desorption ionization-time of flight mass spectrometry) analyses, samples were mixed 1:1 with 10 mg α-cyano-4-hydroxycinnamic acid matrix/ml in 0.1% trifluoroacetic acid in 30% acetonitrile. The sample (1 μl) was spotted on a MALDI plate and analyzed using an Applied Biosystems Voyager DE-Pro MALDI-TOF MS operated in reflector mode with a delay of 100 ns, acc voltage of 20 KV, and a grid voltage of 71.5%. The sample was irradiated with a nitrogen laser (337 nm) and 100 individual laser shots were collected for each sample.

Construction of a nonpolar *pduP* deletion

Bases 28–1,386 of the *pduP* coding sequence were deleted via a PCR-based method (Miller and Mekalanos 1988). The deletion was designed to leave the predicted translational start and stop signals of all *pdu* genes intact. The following primers were used for PCR amplification of the flanking regions of *pduP* : primer 1, 5′-GCTCTAGACCAGGCCAACATCATCCGTGAAGTTAG-3′; primer 2, 5′-TCATCGCGACCTCAGCAGGGTTTCGAGTTCAGAAGTAATCATTG-3′; primer 3, 5′-CGCTAACTGAGGTCGCGATGAATACC-3′; and primer 4, 5′-GAAGAGCTCAATTCTGCGGCGGTACGCTGACCACC-3′. Primers 1 and 2 were used to amplify 496 DNA bases upstream of *pduP*, and primers 3 and 4 were used to amplify 508 DNA bases downstream of *pduP*. The upstream and downstream amplification products were purified then fused by a PCR reaction that included 1 ng of each product/μl and primers 1 and 4. The fused product was cut with *Xba*I and *Sac*I (these sites were designed into primers 1 and 4, respectively), and ligated to suicide vector pCVD442 (Miller and Mekalanos 1988) that had been similarly cut. The ligation mixture was used to transform *E. coli* S17.1 by electroporation and transformants were selected on LB medium supplemented with Amp (100 μg/ml). Four transformants were screened by restriction analysis and all released an insert of the expected size (1,004 bp). One of these transformants was used to introduce the *pduP* deletion into the *S. enterica* chromosome using the procedure of Miller and Mekelanos (1988) with the following modification. For the conjugation step, strain BE47 was used as the recipient and exconjugants were selected by plating on LB agar supplemented with Amp (100 μg/μl) and chloramphenicol (20 μg/μl). Deletion of the *pduP* coding sequence was verified by PCR using chromosomal DNA as a template. Lastly, the *thr-480* dCAM insertion used for selection of exconjugants was crossed-off by P22 transduction using a phage lysate prepared with the wild-type strain and by selecting for prototrophy on NCE glucose minimal medium.

Antibody preparation

His_8-PduP purified using Ni^{2+}-affinity chromatography was resolved on a 12% Tris-HCl SDS-PAGE gel. The protein band corresponding to His_8-PduP was excised and used as a source of antigen for the preparation of polyclonal antibody in a New Zealand white rabbit by Cocalico Biologicals (Reamstown, Penn., uSA). To eliminate antibodies cross-reacting with *E. coli* proteins, the antiserum was preadsorbed using acetone powder prepared from *E. coli* BL21DE3 RIL containing pTA925 (the expression strain lacking *pduP*) as described previously (Harlow and Lane 1988).

Western blots

Cultures were grown and prepared for SDS-PAGE and electroblotting as previously described (Havemann et al. 2002). Membranes were probed using adsorbed anti-PduP antiserum (diluted 1:3,500 in blocking buffer) as the source of the primary antibody, and goat anti-rabbit conjugated to alkaline phosphatase (diluted 1:3,000 in blocking buffer) as the secondary antibody. Chromogenic developing agents were used following the manufacturer's instructions (Bio-Rad).

Electron microscopy

For electron microscopy, cells were grown on minimal medium supplemented with 37 mM succinate and 26 mM propanediol. Cultures (10 ml) were incubated in 125-ml Erlenmeyer flasks at 37 °C, with shaking at 275 rpm in a New Brunswick C24 incubator shaker.

For immunogold localization of the PduP protein, cells were prepared as previously described (Bobik et al. 1999). The source of the primary antibody was rabbit polyclonal anti-PduP antisera or preimmune serum diluted in 1:100 in phosphate-buffered saline (PBS). The secondary antibody used was goat anti-rabbit IgG conjugated to12-nm colloidal gold (Jackson ImmunoResearch Laboratories, West Grove, Penn., USA) diluted in 1:30 in PBS.

DNA sequencing and analysis

DNA sequencing was carried out at the University of Florida Interdisciplinary Center for Biotechnology Research DNA Sequencing Core Facility using Applied Biosystems automated sequencing equipment (Perkin Elmer, Norwalk, Conn., USA) or at the University of Florida, Department of Microbiology and Cell Science DNA Sequencing Facility using a LI-COR model 4000L DNA sequencer, automated sequencing equipment, and Base ImagIR Analysis Software version 04.1 h (LI-COR, Lincoln, Neb., USA). The template for DNA sequencing was plasmid DNA purified using Qiagen 100 tips or Qiagen mini-prep kits. BLAST software was used for sequence similarity searching (Altschul et al. 1997).

Chemicals and reagents

Formaldehyde, (*R, S*)-1,2-propanediol, and antibiotics were from Sigma (St. Louis, Mo., USA). Isopropyl-β-D-thiogalactopyranoside (IPTG) was from Diagnostic Chemicals Limited (Charlotteville P.E.I., Canada). Restriction enzymes were from New England Biolabs (Beverly, Mass., USA) or Promega (Madison, Wis., USA). T4 DNA ligase was from New England Biolabs. Glutaraldehyde was from Tousimis (Rockville, Md., USA), and uranyl acetate was from EM Sciences (Washington, Penn., USA). LR White resin was from Ted Pella (Redding, Calif., USA). Acrylamide, agarose, ammonium persulfate, Coomassie Brilliant Blue R-250, EDTA, ethidium bromide, 2-mercaptoethanol, *N, N*-bis-methylene-acrylamide,

powdered milk, SDS and TEMED were from Bio-Rad (Hercules, Calif., USA). Bacterial Protein Extraction Reagent II (BPER-II) was from Pierce (Rockford, Ill., USA). Other chemicals were from Fisher Scientific (Pittsburgh, Penn., USA).

Results

Effect of a precise *pduP* deletion on the growth of *S. enterica* on minimal 1,2-propanediol medium

Prior biochemical studies indicated that a CoA-acylating propionaldehyde dehydrogenase was involved in the degradation of 1,2-propanediol by *S. enterica* (Toraya et al. 1979; Obradors et al. 1988). Recent DNA sequence analyses of the *pdu* operon (a cluster of genes required for 1,2-propanediol degradation by *S. enterica*) showed that PduP has sequence similarity to a number of CoA-acylating aldehyde dehydrogenases and is 32% identical in amino acid sequence to the *E. coli* aldehyde/alcohol dehydrogenase, AdhE (Kessler et al. 1991; Bobik et al. 1999). This suggested that PduP is a CoA-acylating propionaldehyde dehydrogenase needed for the degradation of 1,2-propanediol by *S. enterica*.

To examine the role of PduP in 1,2-propanediol degradation, a precise deletion of *pduP* was constructed using a PCR-based method, and the effects of this mutation on the growth of *S. enterica* on 1,2-propanediol/CN-B_{12} minimal medium were examined. The wild-type strain and strain BE191 (*ΔpduP*) grew with generation times of about 7.4 and 21.7 h, respectively, and reached maximum optical densities at 600 nm of about 1.38 and 0.49, respectively. This showed the *ΔpduP* mutant was significantly impaired for growth on 1,2-propanediol/CN-B_{12} minimal medium.

As a control, we tested whether the growth defect of strain BE191 (*ΔpduP*) could be corrected by the expression of *pduP in trans*. The generation times of strains BE270 (*ΔpduP*/pLAC22-*pduP*) and BE269 (*ΔpduP*/pLAC22-no insert) on 1,2-propanediol/CN-B_{12} minimal medium were 3.2 and 25.4 h, respectively, and the maximum optical densities reached by these strains were 1.7 for BE270 and 0.45 for BE269. Hence, expression of *pduP in trans* corrected the growth defect of the *ΔpduP* mutation on 1,2-propanediol/CN-B_{12} minimal medium. This result indicated that the observed growth defect of strain BE191 was due to deletion of *pduP*, but not to polarity or a mutation inadvertently introduced during strain construction. This was the expected result since strain BE191 was constructed by a PCR-based method designed to produce a precise nonpolar deletion. Strain BE270 was found to grow about 2.2-fold faster than the wild-type strain. It appeared that pLAC22 enhanced growth of this strain on 1,2-propanediol minimal medium for unknown reasons, since the wild-type strain also grew faster when it carried this vector without insert (not shown). Growth tests were repeated twice with similar results. The fact that strain BE191 (*ΔpduP*) was impaired for growth on 1,2-propanediol/CN-B_{12} minimal medium, in conjunction with the control experiments described above indicates that *pduP* plays an important role in 1,2-propanediol degradation in vivo.

Propionaldehyde dehydrogenase activity in the wild-type and a *ΔpduP* mutant

To test whether *pduP* encodes a propionaldehyde dehydrogenase, enzyme assays were carried out on cell extracts of wild-type *S. enterica* and strain BE191 (*ΔpduP*). The *ΔpduP* mutant used was the same strain used for the growth studies described above, and assays were conducted on both the supernatants and the pellets that resulted from centrifugation of cell extracts at 31,000×*g* (Table 2). For the wild-type strain, the majority of the propionaldehyde dehydrogenase activity was found in the 31,000×*g* pellet, which had a specific activity 1.15 μmol min^{-1} mg^{-1}. Similar extracts from the *pduP* mutant had only 1% of the specific activity of the wild-type strain (0.011 μmol min^{-1} mg^{-1}). When the substrates propionaldehyde, NAD^+ or HS-CoA were omitted from the assay mixture, no activity was detected. To our knowledge, this was the first experimental evidence that *pduP* encodes a CoA-acylating propionaldehyde dehydrogenase.

It was of interest that the majority of the propionaldehyde dehydrogenase activity was found in the 31,000×*g* pellet from the wild-type strain (99%). Prior studies showed that polyhedral bodies are involved in Ado-B_{12}-dependent 1,2-propanediol degradation by *S. enterica* (Bobik et al. 1999; Havemann et al. 2002). Hence, the finding that the PduP aldehyde dehydrogenase was associated with the 31,000×*g* pellet suggested that it might be associated with the polyhedral bodies which pellet under similar centrifugation conditions (unpublished results).

High-level production of PduP

E. coli strain BE273 (pET41a-His_8-*pduP*) was constructed to produce high levels of recombinant His_8-PduP protein.

Table 2 Propionaldehyde dehydrogenase activity in extracts from wild-type *S. enterica* and strain BE191 (*ΔpduP*). Supernatant (S) and pellet (P) fractions were assayed following centrifugation at 31,000×*g*. *ND* None detected

Extract	S/P	Total protein (mg)	Propionaldehyde dehydrogenase activity		
			Specific activity (μmol min^{-1} mg^{-1})	Total activity (μmol min^{-1})	Total activity (%)
Wild-type	S	45	0.004	0.18	1
	P	25	1.15	29.23	99
Strain BE191 (*ΔpduP*)	S	51	ND	ND	0
	P	30	0.011	0.33	100

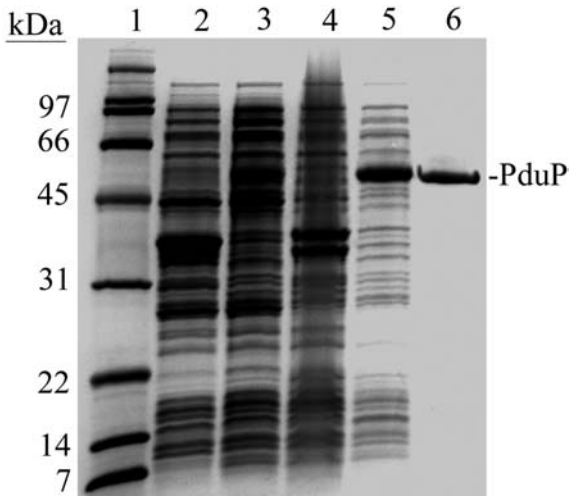

Fig. 2 SDS-PAGE analysis of His_8-PduP production. *Lane 1* Molecular mass markers, *lane 2* 12 µg soluble extract from the control strain (BE237), *lane 3* 12 µg soluble extract from the His_8-PduP expression strain (BE273), *lane 4* 12 µg inclusion-body extract from the control strain, *lane 5* 5 µg inclusion-body extract from the His_8-PduP expression strain, *lane 6* 2 µg His_8-PduP purified by nickel-affinity chromatography. The control and the expression strains were isogenic except that the control strain lacked the *pduP* coding sequence

Protein production by this strain as well as by the control strain BE237 (which is isogenic to BE273 except that it contains the expression plasmid without insert) was analyzed by SDS-PAGE (Fig. 2). Both the soluble and the inclusion-body fractions of cell extracts were examined. High amounts of a protein with a molecular mass near 50 kDa were found in the inclusion-body fraction of cell extracts from expression strain BE273, and moderate amounts of a 50-kDa protein were also found in the soluble fraction of cell extracts from this strain (Fig. 2, lanes 3, 5). This is near the predicted molecular mass of His_8-PduP (50 kDa). In contrast, the soluble and inclusion-body fractions from control strain BE237 (expression vector without insert) contained relatively little protein of 50 kDa molecular mass (Fig. 2, lanes 2, 4) indicating that the observed 50-kDa protein produced by BE273 was His_8-PduP.

The cell extracts analyzed by SDS-PAGE (Fig. 2) were tested for propionaldehyde dehydrogenase activity. Substantial activity (2.4 µmol min^{-1} mg^{-1}) was found in inclusion bodies isolated from strain BE273, and in the soluble fraction of this strain (0.19 µmol min^{-1} mg^{-1}). However, little activity was found in either the soluble or inclusion-body fractions of control strain BE237 (0.02 µmol min^{-1} mg^{-1}). For the inclusion-body fraction from the expression strain, propionaldehyde dehydrogenase activity was linear with protein concentration (data not shown). Furthermore, when the substrates propionaldehyde, NAD or HS-CoA were omitted from the assay mixture, no activity was detected. Thus, these data provided additional evidence that PduP is a CoA-acylating propionaldehyde dehydrogenase.

Purification of recombinant His_8-PduP protein

It was observed that following storage of purified inclusion bodies at 4 °C for 24 h a significant amount of active His_8-PduP protein was extracted into the soluble fraction. Following centrifugation of this preparation at 31,000×*g* for 30 min, approximately 0.5 mg soluble protein remained in the supernatant. Enzyme assays showed that this supernatant contained 9.22 µmol min^{-1} mg^{-1}propionaldehyde dehydrogenase activity. This sample was further purified by nickel-affinity chromatography. The purity of the resulting preparation was analyzed by SDS-PAGE followed by staining with Coomassie. Results indicated that the His_8-PduP obtained was highly purified (Fig. 2, lane 6) and enzyme assays showed that the preparation was enriched in propionaldehyde dehydrogenase activity, with a specific activity of 15.2 µmol min^{-1} mg^{-1}. The reaction requirements for purified PduP were the same as those described above for the partially purified enzyme. These results demonstrated that the His_8-PduP protein had propionaldehyde dehydrogenase activity.

Propionyl-CoA is a product of the PduP reaction

The fact that HS-CoA was required for the propionaldehyde dehydrogenase activity of PduP suggested that propionyl-CoA was a reaction product. Several additional experiments were conducted to test this possibility. Propionaldehyde dehydrogenase reactions containing purified PduP protein were allowed to proceed to completion. Then, reverse-phase HPLC was used to identify HS-CoA and CoA-derivatives present in reaction mixtures. A single CoA compound with a retention time of 10.2 min was detected. Further analyses showed that this compound co-eluted with authentic propionyl-CoA following co-injection and resolution via C-18 reverse-phase HPLC. This indicated that propionyl-CoA was a product of the PduP aldehyde dehydrogenase reaction. Next, the reaction product identified as propionyl-CoA by reverse-phase HPLC was analyzed by MALDI-TOF MS. Three major peaks at *m*/*z* 824.8, 848.1, and 878.1 were observed in the mass spectrum. These peaks correspond to the $[M+H]^+$, $[M+H+Na]^+$, and $[M+3NH_4]^+$ of propionyl-CoA, further supporting the assignment of propionyl-CoA as a PduP reaction product. In addition, the propionaldehyde dehydrogenase reaction catalyzed by the PduP enzyme was followed spectrophotometrically at 232 nm, the wavelength at which thioester bonds characteristically absorb (Dawson et al. 1969). Within experimental error, the specific activity of purified PduP was that same when reactions were followed at 340 nm (NAD reduction) or 232 nm (thioester bond formation). Thus, based on the evidence described above, we conclude that propionyl-CoA is a product of the PduP propionaldehyde dehydrogenase reaction.

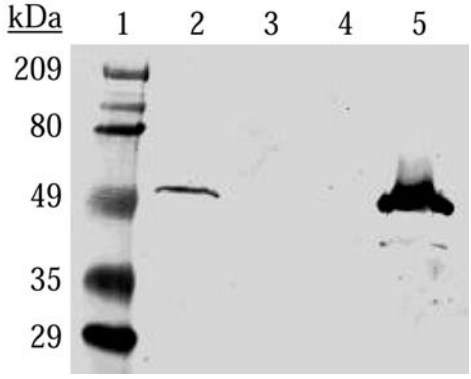

Fig. 3 Western blot using anti-PduP antisera. *Lane 1* Molecular mass standards, *lane 2* wild-type (*Salmonella enterica*), *lane 3* *ΔpduP* (strain BE191), *lane 4* *ΔpduP*/pLAC22 (vector without insert); lane 5, *ΔpduP*/pLAC22-*pduP*. Each lane was loaded with 20 µl boiled whole cells (OD_{600}=1.0)

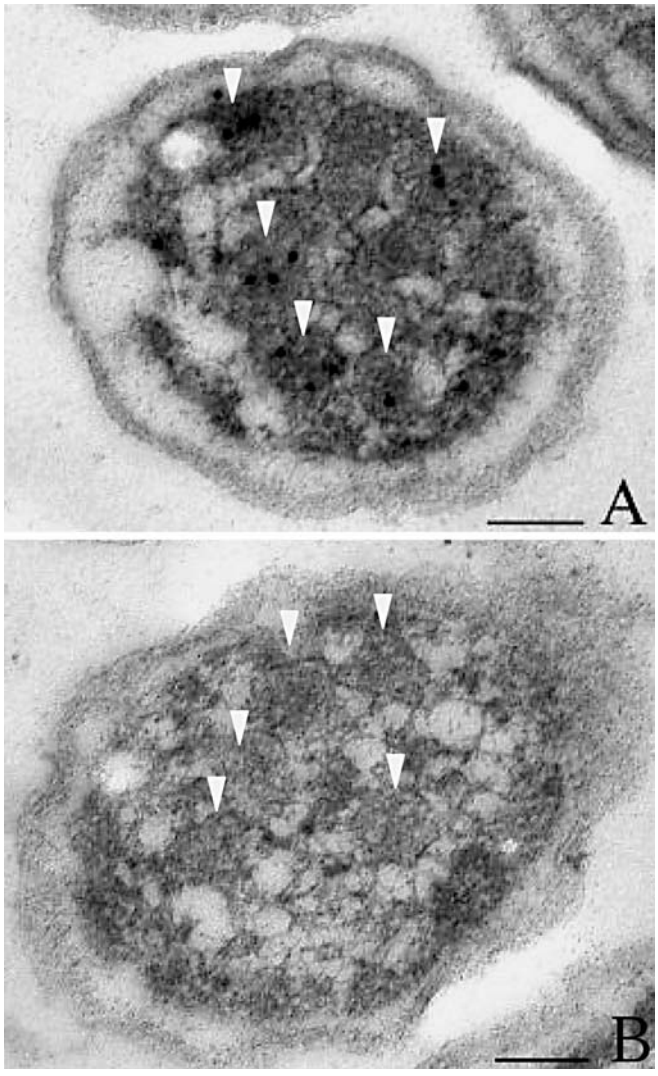

Fig. 4A,B Immunogold localization of the PduP enzyme. **A** Wild-type *S. enterica*; **B** strain BE191 (Δ*pduP*). The 12-nm gold particles (*small black circles*) indicate the location of the PduP protein. The *arrows* point to the polyhedral bodies. *Bars* 100 nm

Preparation and specificity of the anti-PduP antiserum

To obtain the antigen needed for preparation of antisera, His_8-PduP was purified from inclusion bodies isolated from expression strain BE273 by Ni^{2+}-affinity chromatography under denaturing conditions. SDS-PAGE indicated that the PduP was highly purified (data not shown). Denaturing conditions were used to obtain antigen because a nondenaturing protocol was unavailable at the time. Following purification, His_8-PduP was used to prepare polyclonal anti-PduP antiserum in rabbit. The antiserum was subjected to a preadsorbtion procedure to remove cross-reacting proteins (Warren et al. 2000). To determine the specificity of the adsorbed anti-PduP antiserum, Western blots were done on boiled cell lysates. The anti-PduP antibody preparation recognized one major protein band in extracts from wild-type *S. enterica* at approximately 50 kDa (Fig. 3, lane 2); however, this band was not detected in strain BE191, which contained a nonpolar *pduP* deletion but was otherwise isogenic to the wild-type strain (Fig. 3, lane 3). This indicated that the antisera was specific for PduP. Furthermore, the anti-PduP antibody preparation detected a 50-kDa band in boiled cell extracts from PduP expression strain BE270 (pLAC22-PduP) but not in extracts from strain BE269, which carries the pLAC22 expression plasmid without the insert but is otherwise isogenic to strain BE270 (Fig. 3, lanes 4, 5). This provided additional evidence that the antibody preparation was specific for PduP. The minor band observed in Fig. 3, lane 5, was most likely a degradation product of PduP that resulted from overproduction since this band was not detected in extracts from the wild-type strain (Fig. 3, lane 2).

Localization of PduP immunoelectron microscopy

Wild-type *S. enterica* and strain BE191 (*ΔpduP*) were grown on succinate/1,2-propanediol minimal medium, which induces formation of the polyhedral bodies involved in 1,2-propanediol degradation (Bobik et al. 1999; Havemann et al. 2002). Immunogold labeling of wild-type *S. enterica* and strain BE191 (*ΔpduP*) was then carried out using anti-PduP antiserum described above. In the micrograph of Fig. 4), the antibody-conjugated gold particles (solid black circles) indicate the location of the PduP protein. Note that the gold particles localized to the polyhedral bodies (which appear as uniformly stained regions within the cytoplasm) in the wild-type strain, but no labeling was observed in the *ΔpduP* mutant, although polyhedra were present. These results indicated that the antibody preparation reacted specifically with the PduP protein under the labeling conditions used and that PduP localizes to the polyhedral bodies. Additional electron microscopy studies of standard thin sections showed that the *ΔpduP* mutant formed normal-appearing polyhedra (standard thin sections result in higher contrast than does fixation for immunolabeling and the fine structure of the polyhedra is more easily discerned). This result indicated that PduP is nonessential for polyhedral body formation.

Table 3 Propionaldehyde dehydrogenase activity during polyhedral body purification

Sample	Protein (mg)	total activity (μmol min^{-1})	specific activity (μmol min^{-1} mg^{-1})	Yield (%)	-Fold purification
Crude extract	515	152	0.30	100	1.0
Detergent/salts treatment	338	205	0.61	135	2.1
12,000×*g* super	338	169	0.50	111	1.7
48,000×*g* pellet	5	9.6	1.92	6.3	6.5
12,000×*g* super	4.3	8.6	2.00	5.7	6.8
Sucrose density gradient	0.3	1.2	4.00	0.8	13.5

Propionaldehyde dehydrogenase activity is associated with purified *pdu* bodies

To further investigate the subcellular localization of the PduP enzyme, the polyhedral bodies involved in 1,2-propanediol degradation were purified and propionaldehyde dehydrogenase activity was followed during the course of their purification (Table 3). The polyhedra were purified by a combination of detergent treatment and differential and density-gradient centrifugation as described in Havemann and Bobik (2003). Electron microscopy and SDS-PAGE indicated that the polyhedra obtained were highly purified (not shown). During the purification, the specific activity of the PduP propionaldehyde dehydrogenase increased 13.5-fold, from 0.3–4.0 μmol min^{-1} mg^{-1} protein (Table 3). This suggested that the polyhedral bodies comprised about 7% of the total cell protein, which is consistent with previous electron microscopy (Havemann et al. 2002). Furthermore, Western blots verified that the PduP enzyme was associated with the purified polyhedra (not shown). Thus, enzyme assays and Western blots with purified polyhedra supported the immunolabeling studies described above and indicated that the PduP propionaldehyde dehydrogenase is associated with the polyhedral bodies involved in 1,2-propanediol degradation.

Discussion

Prior biochemical studies indicated that a CoA-acylating propionaldehyde dehydrogenase was required for Ado-B_{12}-dependent 1,2-propanediol degradation (Toraya et al. 1979; Obradors et al. 1988). Subsequent DNA sequence analyses of the *pdu* operon identified a presumptive enzyme (PduP) that was 32% identical to the *E. coli* aldehyde/alcohol dehydrogenase, AdhE (Bobik et al. 1999). In this report, genetic and biochemical evidence was presented that established PduP as a CoA-acylating propionaldehyde dehydrogenase involved in Ado-B_{12}-dependent 1,2-propanediol degradation by *S. enterica*.

Previously, Escalante's group proposed that two propionaldehyde dehydrogenases are involved in 1,2-propanediol degradation by *S. enterica*: one that produces propionyl-CoA and a second that oxidizes propionaldehyde directly to propionate (Palacios et al. 2003). The PduP enzyme studied here produced propionyl-CoA as a reaction product and was inactive in the absence of HS-CoA, showing that it is incapable of oxidizing propionaldehyde directly to propionate under the assay conditions used. It was also shown that an *S. enterica* strain with a nonpolar *pduP* null mutation grew at one-third the rate of the wild-type strain. The residual growth of a *ΔpduP* mutant could have resulted from the activity of an alternative propionaldehyde dehydrogenase, such as the one proposed to convert propionaldehyde directly to propionate (Palacios et al. 2003), since the propionate produced by this enzyme could be used as a carbon and energy source via the methyl citrate pathway (Horswill and Escalante-Semerena 1997).

Previously, we established that *S. enterica* formed polyhedral bodies during Ado-B_{12}-dependent growth on 1,2-propanediol (Bobik et al. 1999). Immunolabeling studies demonstrated that these bodies consisted of a protein shell (partly composed of the PduA protein) B_{12}-dependent diol dehydratase and additional unidentified proteins (Bobik et al. 1999; Havemann et al. 2002). In this report, we presented several lines of evidence that indicated PduP is polyhedral-body-associated. The finding that the *pdu* polyhedra include both diol dehydratase (aldehyde-producing) and propionaldehyde dehydrogenase (aldehyde-consuming) is consistent with the prior proposal that these structures function to minimize aldehyde toxicity (Chen et al. 1994; Stojiljkovic et al. 1995; Havemann et al. 2002). Presumably, the shell of the polyhedral body could trap the propionaldehyde produced by the PduCDE diol dehydratase until it is consumed by the PduP propionaldehyde dehydrogenase. Sequestering propionaldehyde until it is converted to propionyl-CoA might protect sensitive cytoplasmic components. Alternatively, the inclusion of diol dehydratase and propionaldehyde dehydrogenase in the *pdu* polyhedra might simply serve to juxtapose these enzymes in order to facilitate propionaldehyde channeling, although it is unclear why such a complex structure would be needed for this sole purpose. Prior studies suggested that the *pdu* polyhedra minimize aldehyde toxicity by limiting aldehyde production through control of Ado-B_{12} availability (Havemann et al. 2002). Such a mechanism would be improved by either aldehyde channeling or sequestration, and a combined mechanism that fine tunes propionaldehyde production and consumption could explain the selective advantage provided by these complex polyhedral bodies involved in 1,2-propanediol degradation.

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