PduA Is a Shell Protein of Polyhedral Organelles Involved in Coenzyme B_{12}-Dependent Degradation of 1,2-Propanediol in *Salmonella enterica* Serovar Typhimurium LT2†

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Salmonella enterica forms polyhedral organelles involved in coenzyme B_{12}-dependent 1,2-propanediol degradation. These organelles are thought to consist of a proteinaceous shell that encases coenzyme B_{12}-dependent diol dehydratase and perhaps other enzymes involved in 1,2-propanediol degradation. The function of these organelles is unknown, and no detailed studies of their structure have been reported. Genes needed for organelle formation and for 1,2-propanediol degradation are located at the 1,2-propanediol utilization (*pdu*) locus, but the specific genes involved in organelle formation have not been identified. Here, we show that the *pduA* gene encodes a shell protein required for the formation of polyhedral organelles involved in coenzyme B_{12}-dependent 1,2-propanediol degradation. A His$_6$-PduA fusion protein was purified from a recombinant *Escherichia coli* strain and used for the preparation of polyclonal antibodies. The anti-PduA antibodies obtained were partially purified by a subtraction procedure and used to demonstrate that the PduA protein localized to the shell of the polyhedral organelles. In addition, electron microscopy studies established that strains with nonpolar *pduA* mutations were unable to form organelles. These results show that the *pduA* gene is essential for organelle formation and indicate that the PduA protein is a structural component of the shell of these organelles. Physiological studies of nonpolar *pduA* mutants were also conducted. Such mutants grew similarly to the wild-type strain at low concentrations of 1,2-propanediol but exhibited a period of interrupted growth in the presence of higher concentrations of this growth substrate. Growth tests also showed that a nonpolar *pduA* deletion mutant grew faster than the wild-type strain at low vitamin B_{12} concentrations. These results suggest that the polyhedral organelles formed by *S. enterica* during growth on 1,2-propanediol are not involved in the concentration of 1,2-propanediol or coenzyme B_{12}, but are consistent with the hypothesis that these organelles moderate aldehyde production to minimize toxicity.

Salmonella enterica degrades 1,2-propanediol in a coenzyme B_{12}-dependent manner (13). The genes needed for this process were acquired by a horizontal gene transfer that is thought to be one of several related events important to the divergence of *S. enterica* and *Escherichia coli* (16, 26). In vivo expression technology 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 (7, 11). Moreover, 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 (29).

At first glance, the degradation of 1,2-propanediol appears to be a relatively simple process. The proposed pathway begins with the conversion of 1,2-propanediol to propionaldehyde, a process mediated by coenzyme B_{12}-dependent diol dehydratase (21, 37). The aldehyde is then converted to propanol and propionic acid, presumably by alcohol dehydrogenase, coenzyme A-dependent aldehyde dehydrogenase, phosphotransacylase, and propionate kinase. This pathway generates one ATP, an electron sink, and a 3-carbon intermediate (propionyl-coenzyme A), which can feed into central metabolism via the methyl citrate pathway (12). In *S. enterica*, the degradation of 1,2-propanediol occurs aerobically, or anaerobically when tetrathionate is supplied as an electron acceptor (26).

The complexity of 1,2-propanediol degradation became apparent when the DNA sequence of the *pdu* locus was determined, and 23 *pdu* genes were identified: 6 *pdu* genes are thought to encode enzymes needed for the 1,2-propanediol degradative pathway; 2 are involved in transport and regulation; 2 are probably involved in diol dehydratase reactivation; 1 is needed for the conversion of vitamin B_{12} (CN-B_{12}) to coenzyme B_{12}; 5 are of unknown function; and 7 share similarity to genes involved in carboxysome formation (6). Carboxysomes are polyhedral organelles found in cyanobacteria and some chemoautotrophs (32, 34, 35). They are composed of a proteinaceous shell that houses most of the cell's ribulose bisphosphate carboxylase/oxygenase (RuBisCO). They are required for autotrophic growth at low CO_2 concentrations and are thought to function as part of a CO_2-concentrating mechanism (15, 23, 25).

Recently, *S. enterica* was shown to form polyhedral organelles that resemble carboxysomes during aerobic and anaerobic growth on 1,2-propanediol (6). These organelles are approximately 150 nm in cross-section and appear to consist of a proteinaceous shell and interior (6). However, there

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TABLE 1. Bacterial strains

Species and strains	Genotype
E. coli	
BE11	(*E. coli* ER2267) F⁻e14⁻ (MrcA⁻) *endA1 supE44 thi-1 relA1? rfbD1? spoT1?* Δ(*mrcC-mrr*) *114*::IS*10* Δ(*argF-lac*)*U169 recA1/F*⁻ *proA*⁺*B*⁺ *lacI*�q Δ(*lacZ*)M15 *zzf*::mini-Tn*10* (Kan^r)/pMGS2
BE229	(*E. coli* ER1992) F⁻λ⁻ Δ(*argF-lac*)*U169 supE44* e14⁻ *dind1*::Mu*d*I1473 (Kan^r *lacZ*⁺) *rtbD1? relA1? endA1 spoT1? thi-1* Δ(*mrcL-mrr*)*114*::IS*10*/pGH48 (His₆-PduA Ap^r)
BE230	BL21(DE3)/pGH48(His₆-PduA Ap^r) and pSJS1240 (*ileX argU* Sp^r)
BE231	BL21(DE3) RIL/pGH107(T7 expression vector with *pduJ* insert, Kan^r)
BL21(DE3)	(*E. coli* B) F⁻ *ompT hsdS*(r_B⁻ m_B⁻) *dcm*⁺ Tet^r *gal* λ (DE3) *endA*
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)
RT1679	DH5α/pVJ70 (contains *pduF'ABCDE ORF1'* Ap^r)
S. enterica serovar Typhimurium LT2	Wild-type
BE33	*pduA672*:: TPOP1
BE39	*pduA673* TPOP1::fragment (a short nonpolar insertion resulting from the spontaneous deletion of the majority of the *pduA672*::TPOP1 element)
BE103	TR6579/pKD46ᵃ
BE182	Δ*pduA652* (precise nonpolar deletion of the *pduA* gene)ᵇ
BE228	Δ*pduA652*/pTA749 (p*lacI*ᵠPO-*Bgl*II with *pduA* insert, Ap^r)
BE232	*metE205 ara-9* Δ*299*/p*lacI*ᵠPO-*Bgl*II (no insert, Ap^r) (in Δ*299* the entire *cob* and *pdu* operons are deleted)
BE233	*metE205 ara-9* Δ*299*/pTA749 (*pduA* under p*lac* control, Ap^r) (in Δ*299* background the entire *cob* and *pdu* operons are deleted)
BE235	*pdu12*::Mu*d*J
TR6579	*metA22 metE551 trpD2 ivl-452 hsdLT6 hsdSA29* HsdB⁻ *strA120* GalE⁻ Leu⁻ Pro⁻

ᵃ See reference 8.
ᵇ See Materials and Methods.

are significant differences between the *S. enterica* organelles and carboxysomes. *S. enterica* is not an autotroph and does not express RuBisCO. The *S. enterica* organelles are involved in coenzyme B₁₂-dependent 1,2-propanediol degradation and are associated with coenzyme B₁₂-dependent diol dehydratase and perhaps other enzymes (6). A role in CO₂ concentration, similar to that of the carboxysome, is uncertain since there is no known association between CO₂ and coenzyme B₁₂-dependent 1,2-propanediol degradation in *S. enterica*. Thus, the function of the *S. enterica* organelles and the extent of their similarity to carboxysomes remain important questions.

Seven genes found in the *pdu* operon have DNA sequence similarity to those required for carboxysome formation, and four of these (*pduAJKT*) encode proteins that have similarity to carboxysome shell proteins (6, 33). The roles of these *pdu* genes in organelle formation have not been investigated, and no genetic studies on organelle function have been reported. Here, we show that the *pduA* gene is required for the formation of polyhedral organelles by *S. enterica*. Results show that the PduA protein is a component of the organelle's shell. Physiological studies revealed that a *pduA* mutant grew similarly to the wild type on minimal medium containing lower concentrations of 1,2-propanediol but exhibited a period of interrupted growth at higher concentrations. This mutant also grew at a faster rate than the wild type at low CN-B₁₂ concentrations, suggesting that the organelle's shell may present a barrier to B₁₂ entrance. These results indicate that the organelles of *S. enterica* are not involved in concentrating 1,2-propanediol or CN-B₁₂, but are consistent with a role in moderating aldehyde toxicity.

MATERIALS AND METHODS

Chemicals and reagents. Formaldehyde (*R,S*), 1,2-propanediol, trichloroacetic acid, and antibiotics were from Sigma Chemical Company (St. Louis, Mo.). Isopropyl-β-D-thiogalactopyranoside (IPTG) was from Diagnostic Chemicals Limited (Charlottetown, Canada). Restriction enzymes were from New England Biolabs (Beverly, Mass.) or Promega (Madison, Wis.). T4 DNA ligase was from New England Biolabs. Electrophoresis supplies were from Bio-Rad (Hercules, Calif.). Bacterial Protein Extraction Reagent II and Micro BCA reagents were from Pierce (Rockford, Ill.). Other chemicals were from Fisher Scientific (Pittsburgh, Pa.).

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 medium (Difco Laboratories, Detroit, Mich.) (20). The minimal medium used was the No-carbon-E (NCE) medium (4, 19, 38). Amino acids were provided at the following concentrations: valine, isoleucine, leucine, and threonine, 0.3 mM; and histidine, 0.1 mM. Antibiotics were provided in liquid or solid rich medium at the following concentrations unless otherwise stated: carbenicillin, 100 μg/ml; ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; spectinomycin, 50 μg/ml; and tetracycline, 20 μg/ml. Ampicillin was used at 15 μg/ml in minimal medium. Tetracycline was used at 2 μg/ml to induce expression out of TPOP insertions. IPTG was added to a final concentration of 1 mM for induction of genes cloned into pET vectors or p*lacI*ᵠPO-*Bgl*II. MacConkey–1,2-propanediol–vitamin B₁₂ indicator plates and aldehyde indicator plates were prepared as described previously (6, 13).

General protein and molecular methods. Plasmid purification, bacterial transformation, electrophoresis, and other standard molecular and protein methods were performed as previously described (14, 30).

P22 transduction and transposon mutagenesis of the *pdu* region. Transductional crosses were performed as described previously (9, 31). For TPOP1 mutagenesis of the *pdu* region, a pool of approximately 80,000 independent insertion mutants was prepared as described previously (5). The pool was then used as a donor in transductional crosses with strain BE235 (*pdu12*::Mu*d*J). Tetracycline resistance was selected, and transductants were screened for loss of the *pdu12*::Mu*d*J elements using MacConkey–lactose–1,2-propanediol indicator plates (5). Following transposon mutagenesis, 30,000 colonies were screened, and 120 TPOP1 insertions located near the *pdu* operon were isolated.

Localization of TPOP1 insertions. PCR was used to amplify the region of DNA that included one join point between the TPOP1 element and the *S.*

enterica chromosome. The primers used for PCR amplification were 5′-ACCTT TGGTCACCAACGCTTTTCC-3′ and 5′-GTTCATATGCGAAACCACTTC-3′. The DNA sequence of the PCR product was determined, and DNA sequence analysis showed that the downstream join point between the *pduA672*::TPOP1 and the *S. enterica* chromosome was bp 160 of the *pduA* coding sequence.

Growth curves. Growth curves were determined as described previously (14). For physiological studies that employed various CN-B$_{12}$ and 1,2-propanediol concentrations, cells were grown in 16- by 100-mm test tubes containing 5 ml of appropriate medium. Cultures were incubated as described for the aerobic growth curves, except the tubes were held in place at an angle of 45°. Cell growth was monitored by measuring the optical density at 600 nm (OD$_{600}$) using a Spectronic 20D$^+$ spectrophotometer. Inocula for the growth curves were prepared as described previously (14), and 0.125 ml was used to inoculate 5-ml cultures.

Cloning of *pduA* for high-level expression. The following primers were used for PCR amplification of *pduA* DNA using pVJ70 (RT1679) as a template: forward, 5′-GGAATTCCATATGCAACAAGCACTAGGAATGG-3′; and reverse, 5′-C ACCGATGGATCCTCATTGGCTAATTCCCTTCG-3′. The PCR product was gel purified, restricted with *Nde*I and *Bam*HI, and ligated to the similarly restricted plasmid pET15b. The ligation reaction mixture was heated to 70°C for 15 min and used to transform *E. coli* ER1992 by electroporation. The DNA sequence of the cloned DNA was shown to be in agreement with the previously reported *pduA* DNA sequence (6). For protein expression, *E. coli* BL21(DE3)/pSJS1240 was used as the host strain.

Cloning of *pduJ*. The following primers were used for PCR amplification of *pduJ* using pMGS2 (BE11) as a template: forward, 5′-GGAATTCATATGAAT AACGCACTGGGACTGG-3′; and reverse, 5′-AGGATCATGCTCGAGGGC TGATTTCGGTAAAATGG-3′. The PCR product was gel purified, restricted with *Nde*I and *Xho*I, and ligated to the similarly restricted plasmid pET41a. The ligation reaction was heated to 70°C for 15 min and used to transform *E. coli* DH5α by electroporation. The sequence of the cloned DNA was shown to be in agreement with the previously reported *pduJ* DNA sequence (6). For protein expression, *E. coli* BL21(DE3) RIL was used as the host strain.

Purification of the PduA protein. A 200-ml culture of BE230 was prepared using LB–ampicillin–spectinomycin–1% glucose medium incubated at 37°C in a 1-liter baffled flask with shaking at 275 rpm. When the cells reached an OD$_{600}$ of 0.8, expression of recombinant His$_6$-PduA protein was induced by addition of 1 mM IPTG. Cells were lysed, and inclusion bodies were isolated by treatment with Bacterial Protein Extraction Reagent II according to the manufacturer's protocol. The inclusion bodies were then solubilized in binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl [pH 7.9], 6 M guanidine hydrochloride) and filtered though a 0.45-μm-pore size cellulose acetate filter. His$_6$-PduA was purified from the solubilized inclusion bodies using a 1-ml Amersham Pharmacia HiTrap Chelating (Ni^{2+}) column. The manufacturer's directions were followed with the following modifications: a 20-ml 20 to 200 mM linear imidazole gradient was used to elute the recombinant His$_6$-PduA, and 6 M guanidine hydrochloride was added to the binding, wash, and elution buffers.

Antibody preparation. His$_6$-PduA protein obtained from Ni^{2+} affinity chromatography (see above) was resolved on a 12% polyacrylamide gel. The portion of the gel containing the PduA protein was excised and used as a source of antigen. Polyclonal antibodies were prepared in a New Zealand White rabbit by Cocalico Biologicals (Reamstown, Pa.).

For subtraction of antibodies reacting with PduJ and *E. coli* proteins, two 200-ml cultures of BE231 were prepared using Luria broth–kanamycin–1% glucose medium incubated at 37°C in 1-liter baffled flasks with shaking at 275 rpm. When the cells reached an OD$_{600}$ of 0.8, expression of recombinant PduJ-His$_8$ protein was induced by addition of 1 mM IPTG. An acetone powder made out of whole cells was used to subtract antibodies reacting with the PduJ protein as described previously (10).

Western blots. Cultures were grown in NCE minimal medium supplemented with 0.4% propanediol, 1% succinate, and the amino acids valine, leucine, isoleucine, and threonine. The pellet from 1 ml of cells was mixed with enough Tris-Tricine loading buffer to obtain an OD$_{600}$ of 1. Samples were boiled for 8 min at 100°C, and 20 μl (equivalent to protein from cells with an OD$_{600}$ of 0.02) was separated on a 16.5% Tris-Tricine gel. Electroblotting was performed in 10 mM morpholineethanesulfonic acid–20% MeOH, pH 6.0, using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) run at a constant 20 V for 14.5 h at 4°C using a PowerPac 1000 power supply (Bio-Rad). The membrane used was Hybond-P (Amersham Pharmacia Biotech, Buckinghamshire, England). Membranes were probed as described previously (10) using anti-PduA polyclonal antibodies diluted 1:7,000 in blocking buffer as the primary antibody and goat anti-rabbit immunoglobulin G-alkaline phosphatase conjugate (Bio-Rad) diluted

1:3,000 in blocking buffer as the secondary antibody. Color developing reagents were used as specified in the manufacturer's directions (Bio-Rad).

Electron microscopy. For electron microscopy, cells were grown on minimal medium supplemented with 1% succinate and 0.4% propanediol. Cultures (10 ml) were incubated in 125-ml shake flasks at 37°C, with shaking at 275 rpm in a New Brunswick C24 Incubator Shaker. For the observation of ultrastructure, cells were sectioned, fixed, observed, and photographed as described previously (6).

For immunogold localization of the PduA protein and diol dehydratase, cells were sectioned, fixed, observed, and photographed as described previously (6). The primary antibodies used were either rabbit polyclonal antibody against the PduA protein (anti-PduA) diluted 1:100 in phosphate-buffered saline (PBS), rabbit polyclonal antibody against diol dehydratase diluted 1:1,000 in PBS, or preimmune serum diluted 1:100 in PBS. The secondary antibody used was goat anti-rabbit immunoglobulin G conjugated to 12-nm colloidal gold (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.) diluted 1:30 in PBS.

Construction of a nonpolar *pduA* deletion. A nonpolar deletion of *pduA* was constructed as described by Datsenko and Wanner (8) with some modifications. The primers used for PCR amplification of the kanamycin resistance cassette from plasmid pKD4 were 5′-GTGTCCCAACTATCGGAACACTCCATGCGA GGTCTTTTGTAGGCTGGAGCTGCTTCG-3′ and 5′-CTGCGCCATGATCT GTTCCACCAGCTCATTGCTGCATGAATATCCTCCTTAGTTC-3′. Strain BE103 was used as the host strain for linear transformation. The primers used to verify the chromosomal location of the kanamycin cassette insertion were 5′-G TCCTGGCCAGCGCAAGTTTCGGC-3′, 5′-CAGTCATAGCCGAATAGCC T-3′, 5′-CGGTGCCCTGAATGAACTGC-3′, and 5′-GCTTTTTCCAGCGCA TAGCTGGCGCGAGC-3′. After the insertion site was verified by PCR, the kanamycin cassette was moved into LT2 via transduction. This cassette was then removed using the FlP recombinase as described previously (8). DNA sequencing showed that the expected deletion was formed; the entire *pduA* coding sequence was deleted except for the last 29 bp, which included the native ribosome binding site of the *pduB* gene.

DNA sequencing and analysis. DNA sequencing was carried out by the University of Florida Interdisciplinary Center for Biotechnology Research, DNA Sequencing Core Facility, as described previously (6) and 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, Nebr.). BLAST software was used for sequence similarity searching (1).

RESULTS

Purification of recombinant His$_6$-PduA protein. *E. coli* strain BE230 was constructed to produce high levels of recombinant His$_6$-PduA protein. Samples of induced and uninduced boiled cells of this strain were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to determine if the His$_6$-PduA protein was being expressed. When induced by IPTG, strain BE230 produced high levels of an 11.8-kDa protein (Fig. 1, lane 3), which was not seen in the uninduced sample (Fig. 1, lane 2). This observed mass correlates well with the predicted mass of 11.4 kDa for the recombinant His$_6$-PduA protein. Electron microscopy revealed that strain BE230 produced rod-like structures in the cytosol when induced with IPTG (data not shown), and SDS-PAGE showed that these inclusion bodies were composed mainly of the His$_6$-PduA protein (Fig. 1, lane 5). Significant amounts of the His$_6$-PduA protein were not found in the soluble fraction (Fig. 1, lane 4).

The His$_6$-PduA protein was purified from inclusion bodies by Ni^{2+} chromatography under denaturing conditions. The His$_6$-PduA protein eluted from the column at approximately 160 to 200 mM imidazole. The fractions containing the His$_6$-PduA protein were analyzed by SDS-PAGE. A single band at the predicted mass for the His$_6$-PduA protein was observed, as well as three faint bands at higher molecular masses (Fig. 1, lane 6). About 600 μg of the partially purified His$_6$-PduA
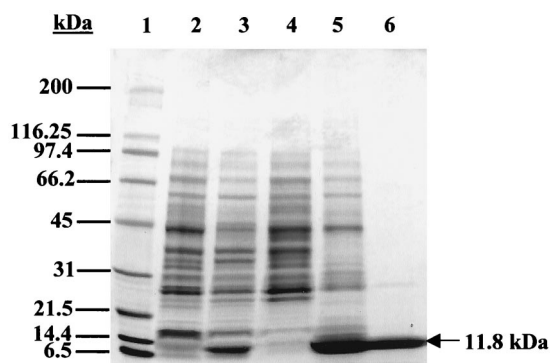
FIG. 1. Overexpression and purification of the His$_6$-PduA protein. Lane 1, molecular mass markers; lane 2, uninduced boiled cell lysate; lane 3, induced boiled cell lysate; lane 4, soluble fraction; lane 5, inclusion body preparation; lane 6, combined fractions 12 to 15 from Ni^{2+} column elution. Molecular masses in kilodaltons are shown at the left.

protein was isolated on a preparatory SDS-PAGE gel, and the band of interest was excised and used as a source of antigen for polyclonal antibody production.

Preparation of PduA-specific antibody. To determine the specificity of the polyclonal antibodies generated against the His$_6$-PduA protein, Western blots were performed on boiled cell lysates. Anti-PduA polyclonal antibodies recognized a major protein band at 9.5 kDa in the wild-type strain but not in strain BE182 (which contains a nonpolar *pduA* deletion mutation) (Fig. 2A, lanes 1 and 2). This indicated that the band at 9.5 kDa corresponded to the native PduA protein. Anti-PduA antibodies also recognized a protein band at 9.5 kDa in strain BE233 (Fig. 2A, lane 4), which carries a plasmid with the *pduA* gene under control of an IPTG-inducible promoter but lacks the entire *pdu* operon due to a deletion mutation. No band at 9.5 kDa was observed in an isogenic strain carrying the same plasmid without an insert (BE232) (Fig. 2A, lane 3). These results confirmed that the band at 9.5 kDa was the native PduA protein.

The anti-PduA antibody preparation also reacted with an 8-kDa protein expressed by the wild-type strain and BE182 (Fig. 2A, lanes 1 and 2) and a 7.5-kDa protein band expressed

by BE233 and BE232 (Fig. 2A, lanes 3 and 4). Additional Western blots of strains overexpressing the PduJ protein indicated that this 8-kDa protein was the PduJ protein (data not shown). The observed 7.5-kDa protein band was apparently plasmid encoded, as this band was detected in Western blots of cells carrying plasmid pTA749 but not in blots of isogenic strains that lacked this plasmid (data not shown). In order to improve the specificity of the polyclonal antibody preparation, a subtraction procedure was performed.

An acetone powder was made from whole cells of strain BE231, a derivative of BL21(DE3) RIL that expressed PduJ at high levels, and then used to subtract antibodies recognizing the PduJ protein and *E. coli* proteins from the anti-PduA polyclonal antibody preparation. After subtraction, the antibody preparation recognized a single band at 9.5 kDa in the wild-type strain (Fig. 2B, lane 1), and no labeling was observed in the *pduA* deletion mutant, BE182 (Fig. 2B, lane 2). The subtracted polyclonal antibody preparation also recognized a 9.5-kDa protein band in strain BE233, which overexpresses the PduA protein (Fig. 2B, lane 4). No labeling at 9.5 kDa was observed in control strain BE232, which carried the plasmid without insert (Fig. 2B, lane 3). These results showed that the subtracted anti-PduA antibody preparation was highly specific for the PduA protein, and this antibody preparation was used for subsequent immunolabeling experiments.

Localization of PduA by immunoelectron microscopy. Immunogold labeling of *S. enterica* cells with highly specific subtracted anti-PduA antibodies (see above) indicated that the PduA protein is a component of the shell of the polyhedral organelles. In the micrograph shown in Fig. 3C, the antibody-conjugated gold particles (solid black circles) indicate the location of the PduA protein. The majority of the gold particles localized to the periphery of the polyhedral organelles. Preimmune serum failed to label the polyhedra (data not shown), and only a small amount of spurious labeling was observed when strain BE182 (Δ*pduA*) was labeled with anti-PduA antibody (Fig. 3D).

Localization of diol dehydratase in a *pduA* mutant. Labeling of wild-type and BE182 (Δ*pduA*) cells with anti-diol dehydratase antibodies illustrated the effect of a nonpolar *pduA* deletion mutation on the localization of diol dehydratase (Fig. 3A and B). In the wild-type strain, diol dehydratase localized to the polyhedral organelles (Fig. 3A), whereas in strain BE182 (Δ*pduA*), which is unable to synthesize polyhedra, diol dehydratase was found in large diffuse aggregates at the poles of the cell (Fig. 3B). This is consistent with the role of the PduA protein as a component of a shell that encases diol dehydratase.

The *pduA* gene is required for the formation of polyhedral organelles. The effect of a *pduA*::TPOP1 insertion mutation on polyhedral organelle formation was investigated. TPOP1 insertions are polar in the absence of tetracycline but nonpolar when growth media are supplemented with this antibiotic (27). Strain BE33 (*pduA672*::TPOP1) exhibited a polyhedron-negative phenotype and failed to grow on 1,2 propanediol–CN-B$_{12}$ minimal medium in the absence of tetracycline. When tetracycline was used to induce downstream expression from the TPOP1 element, BE33 was still unable to synthesize polyhedra, but the ability to grow on 1,2-propanediol minimal medium was restored. This indicated that the *pduA* gene was
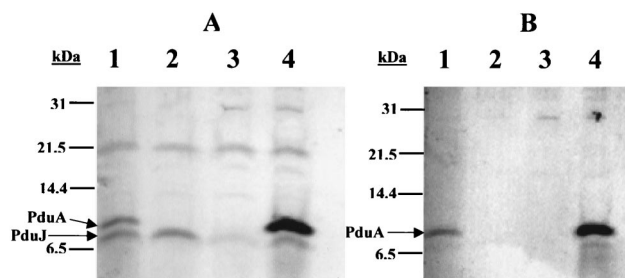

FIG. 2. Western analysis with unsubtracted (A) and subtracted (B) anti-PduA polyclonal antibody preparations. For both panels: lane 1, *S. enterica* serovar Typhimurium LT2; lane 2, BE182 (Δ*pduA* mutant); lane 3, BE232 (isogenic to BE233, except that the expression plasmid lacks an insert); lane 4, BE233 (PduA expression strain). Molecular masses in kilodaltons are shown at the left of each blot. Total protein loaded in each lane was equivalent to that from cells at an OD$_{600}$ of 0.02.
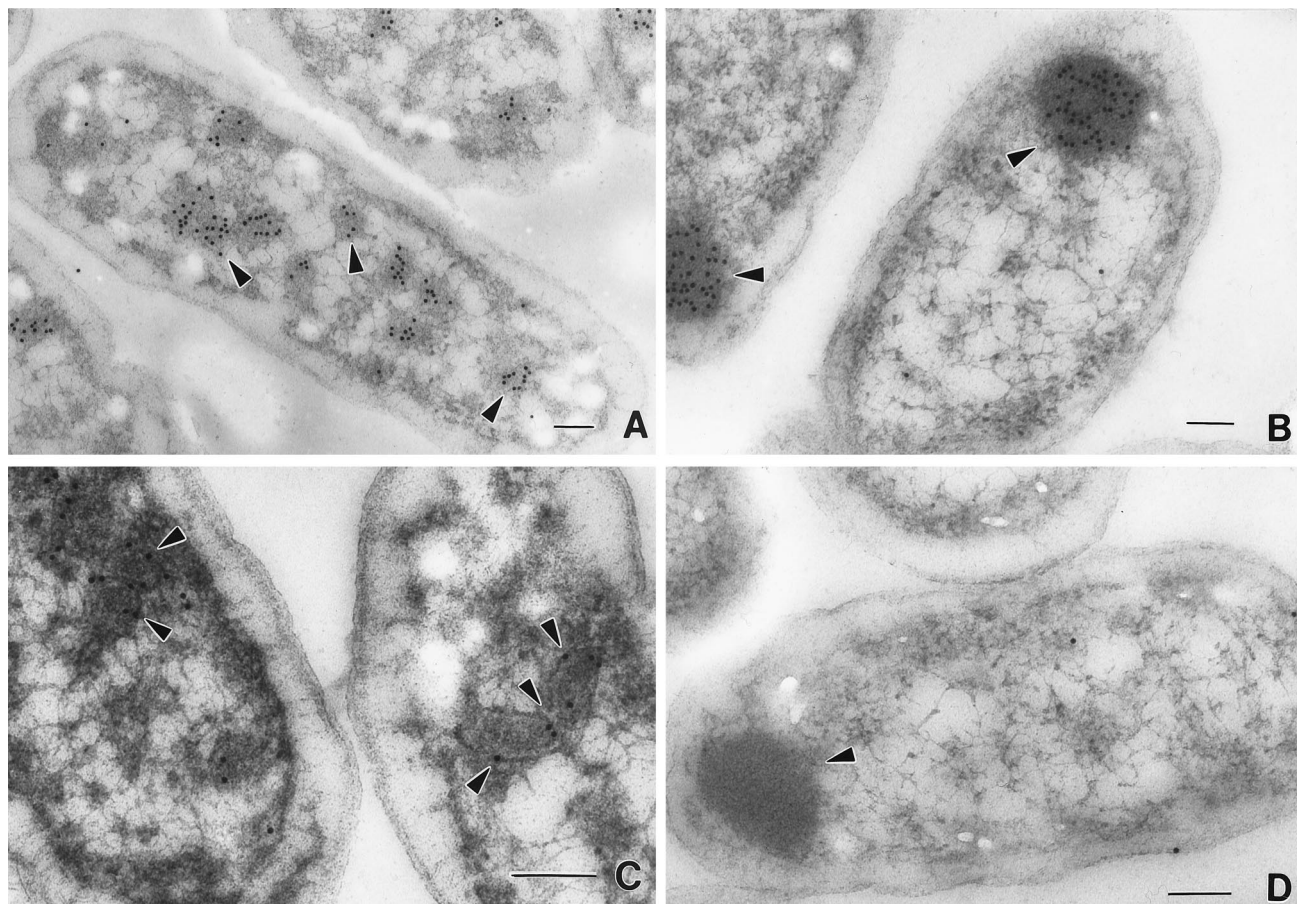
FIG. 3. Localization of diol dehydratase and the PduA protein by immunoelectron microscopy. Cells were grown on succinate minimal medium supplemented with 1,2-propanediol to induce expression of the *pdu* operon. For each panel the arrows point to gold particles that indicate the location of either diol dehydratase or the PduA protein. The strain and protein labeled in each panel are as follows. (A) *S. enterica*, diol dehydratase. (B) BE182 (Δ*pduA*), diol dehydratase. (C) *S. enterica*, PduA. (D) BE182 (Δ*pduA*), PduA. Bars at lower right-hand corners are 100 nm in length.

required for organelle formation but not for growth on 1,2-propanediol. Two additional nonpolar *pduA* mutations were also shown to prevent organelle formation but allow growth on 1,2-propanediol–CN-B$_{12}$ minimal medium. They were BE182 (Δ*pduA*) and BE39 (*pduA*::TPOP1 fragment). These results provided further evidence that the *pduA* gene was required for the formation of polyhedral organelles but not for growth on 1,2-propanediol.

Complementation of the polyhedral organelle-negative phenotype of a Δ*pduA* mutant strain. Strain BE228 contains a nonpolar deletion of the *pduA* coding sequence as well as plasmid pTA749, which allows expression of the PduA protein in response to IPTG. To determine whether the *pduA* gene present on the plasmid could complement the Δ*pduA* mutation for polyhedral body formation, strain BE228 was grown on 1,2-propanediol succinate minimal medium using several IPTG concentrations and then examined by electron microscopy. Strain BE228 synthesized both normal and abnormal polyhedra at 0.01 mM IPTG (Fig. 4A). In the absence of IPTG, no normally shaped organelles were formed and aberrantly shaped structures were rare. The finding that BE228 (Δ*pduA*/pTA749) produced apparently normal organelles in the pres-

ence of IPTG, but not in its absence, showed that the organelle-negative phenotype of strain BE182 was a consequence of the Δ*pduA* mutation. The apparent explanation for the formation of aberrant organelles is that proper organelle assembly required a specific ratio of the PduA protein to other organelle proteins; the prevalence of aberrant polyhedra increased with increasing IPTG concentration (increasing levels of the PduA protein), and at concentrations of ≥0.1 mM, primarily aberrant structures were observed (Fig. 4B). The fact that higher PduA expression levels alters organelle shape supports a structural role for the PduA protein in the formation of the structures.

Strains with *pduA* mutations show a period of interrupted growth when cultured on 1,2-propanediol–CN-B$_{12}$ minimal medium. During aerobic growth on 1,2-propanediol–CN-B$_{12}$ minimal medium, strain BE182 (Δ*pduA*) grew similarly to the wild-type strain except for a period of interrupted growth that initiated at 20 h and persisted until the 40-h mark (Fig. 5). Before the onset of interrupted growth, BE182 (Δ*pduA*) grew with a generation time of 6.8 h, which was slightly faster than the 8.4-h generation time of the wild type-strain. Strain BE182 (Δ*pduA*) also grew slightly faster than the wild type after the
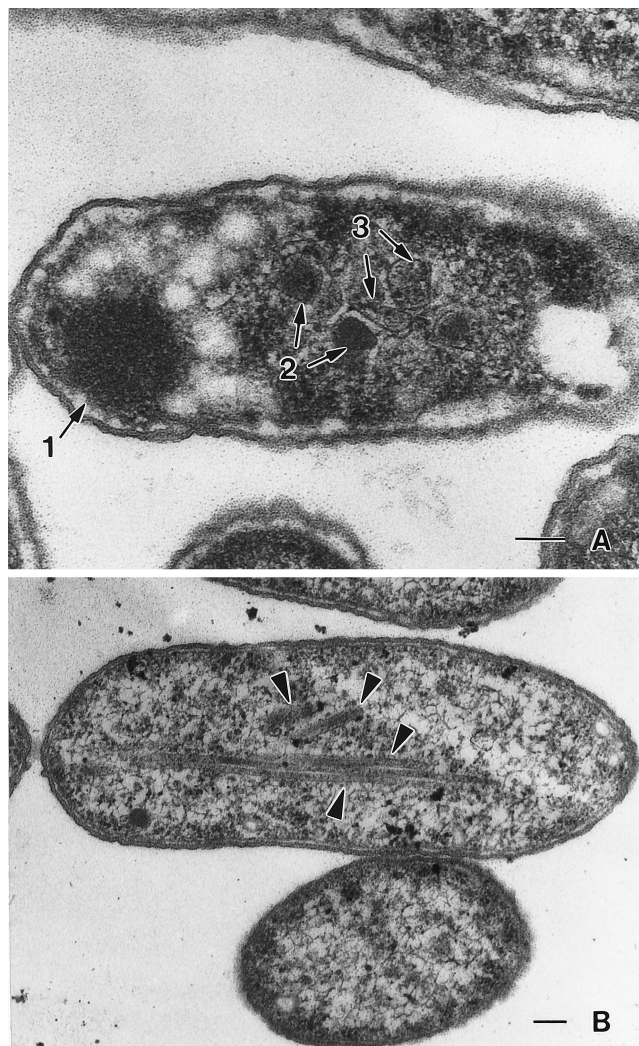
FIG. 4. Complementation of a Δ*pduA* mutation for formation of polyhedral organelles. Strain BE228 (Δ*pduA*/pTA749-PduA expression plasmid) was grown on minimal succinate medium supplemented with 1,2-propanediol and IPTG and then examined by transmission electron microscopy. Strain BE228 was grown with 0.01 (A) and 0.1 (B) mM IPTG, respectively. (A) The numbered arrows indicate the locations of the following structures: 1, polar inclusion body; 2, polyhedral organelles; 3, abnormally shaped organelles. (B) The arrows indicate the aberrant rod-like structures observed in some cells. Bars at the lower right-hand corners are 100 nm in length.

period of interrupted growth; its generation time was 7.7 h, compared to a 9-h generation time for the wild-type strain. The maximum optical density reached by both the wild-type strain and BE182 (Δ*pduA*) was 1.8 at 600 nm. Viable cell counts of the mutant and wild-type strains gave results similar to optical density measurements. The period of interrupted growth was observed in four separate experiments. This phenomenon suggested that a toxic compound was accumulating and inhibiting the growth of *S. enterica* mutants unable to form polyhedral organelles during growth on 1,2-propanediol. Presumably, growth resumed after the induction of genes that mitigated the toxicity problem. Alternatively, interrupted growth could have resulted from the depletion of an essential nutrient. However,
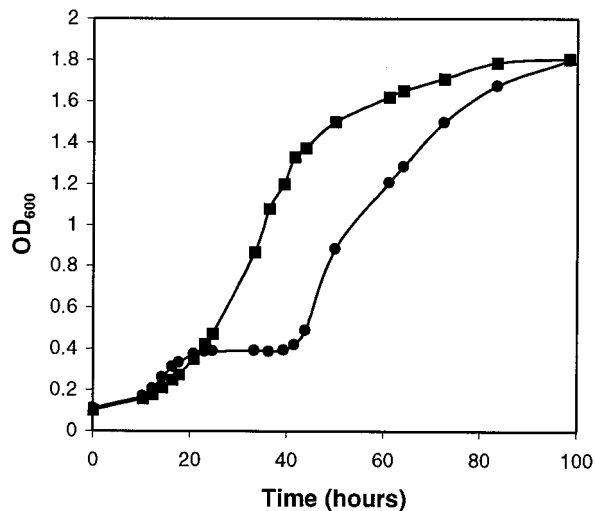

FIG. 5. Growth of the wild-type strain and a *pduA* mutant on 1,2-propanediol–CN-B$_{12}$ minimal medium. Results for the wild type, *S. enterica* serovar Typhimurium LT2 (■), and BE182 (nonpolar Δ*pduA*) (●) are shown. Cells were cultured as described in Materials and Methods.

this seems unlikely, since interrupted growth was dependent on the concentrations of 1,2-propanediol and CN-B$_{12}$ present in the growth medium (see below).

Effects of 1,2-propanediol concentration on the growth rates of wild-type and *pduA* mutant strains. The effects of the 1,2-propanediol concentration on the growth of the wild type and a strain containing a nonpolar *pduA* deletion mutation (BE182) were examined (Fig. 6). At 1,2-propanediol concentrations of 0.01 and 0.05%, the wild-type strain and BE182 grew similarly. The generation times of the wild-type were 10.8 and 11.9 h, respectively, whereas the generation times of BE182 (Δ*pduA*) were 11.7 and 10.7 h, respectively. At higher 1,2-propanediol concentrations, some distinct differences between the wild type and strain BE182 were noted. At 1,2-propanediol concentrations of 0.2 and 0.4%, the wild-type
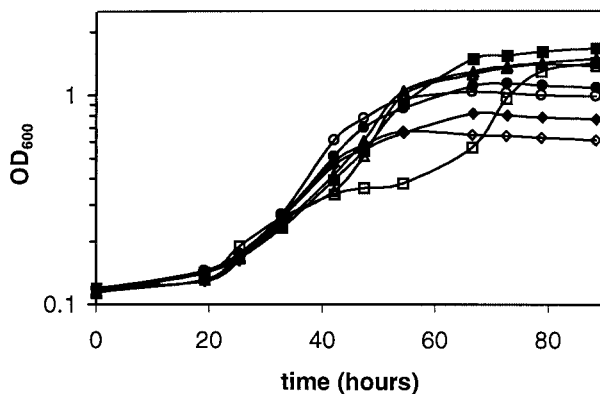

FIG. 6. Effects of various 1,2-propanediol concentrations on the growth of strain BE182 (Δ*pduA*) and the wild-type strain. Cells were cultured on 1,2-propanediol–CN-B$_{12}$ minimal media having the following 1,2-propanediol concentrations: 0.4% (■, □), 0.2% (▲, △), 0.1% (●, ○), and 0.05% (◆, ◇). Closed symbols, wild type, *S. enterica* serovar Typhimurium LT2. Open symbols, BE182 (Δ*pduA*).
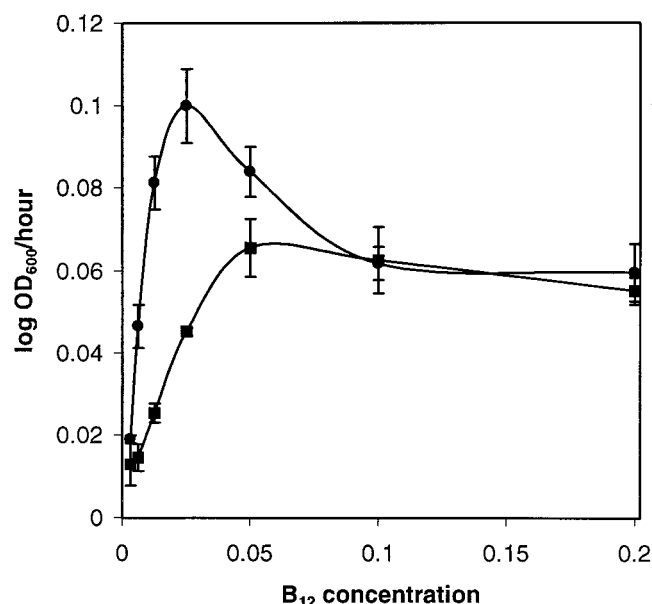
FIG. 7. Effects of various $CN-B_{12}$ concentrations on the growth of strain BE182 ($\Delta pduA$) and the wild-type strain (*S. enterica*). Cells were cultured on 1,2-propanediol minimal medium supplemented with CN-B_{12} at the concentrations indicated. ■, wild-type; ●, BE182 ($\Delta pduA$). Error bars represent one standard deviation.

strain grew with generation times of 10.2 and 12.6 h. At similar 1,2-propanediol concentrations, strain BE182 grew with generation times of 14.5 and 20.3 h followed by a period of interrupted growth and then much shorter generation times of 8 and 10.2 h. In strain BE182 ($\Delta pduA$), the duration of interrupted growth was shorter at a 1,2-propanediol concentration of 0.2% (10 h) than at 0.4% (20 h). Thus, interrupted growth was observed at higher concentrations but not at lower concentrations of 1,2-propanediol. This suggested that interrupted growth resulted from the accumulation of a toxic compound derived from 1,2-propanediol. Propionaldehyde was a likely candidate, since this compound is an intermediate of 1,2-propanediol degradation and aldehydes are well known to have toxic effects on cells. Amino acid supplementation was not used in this experiment, as very slight growth occurred on the amino acids alone. Consequently, the generation times observed are longer than those shown in Fig. 5.

Effects of CN-B_{12} concentration on the growth rates of wild-type and *pduA* null mutant strains. The effects of the CN-B_{12} concentration on the growth of the wild type and a strain containing a nonpolar *pduA* deletion mutation (BE182) were examined (Fig. 7). Strain BE182 ($\Delta pduA$) grew faster than the wild type at concentrations of CN-B_{12} from 0.003125 to 0.05 μg/ml; the largest difference was seen at a concentration of 0.00625 μg/ml, where the generation times were 47.5 h for the wild-type strain and 14.9 h for BE182 ($\Delta pduA$). At CN-B_{12} concentrations of 0.1 and 0.2 μg/ml, BE182 ($\Delta pduA$) reached a maximal growth rate similar to that of the wild-type strain but exhibited the aforementioned interrupted growth (see above). These findings are consistent with a model in which an intermediate of 1,2-propanediol degradation has a toxic effect that interrupts growth. At higher CN-B_{12} concentrations, 1,2-propanediol would be catabolized at a faster rate and the toxic

compound would accumulate to higher levels. This would result in a more pronounced interruption of growth. Subsequently, genes that lessen the effects of the toxic compound would be induced, and the growth rate would increase. In addition, the above results indicate that the polyhedral organelles act as a barrier to B_{12}. The possible significance of this finding is addressed in the Discussion section. In this experiment, amino acid supplementation was not used, as slight growth occurs on the amino acids alone.

DISCUSSION

Previously we showed that *S. enterica* formed polyhedral organelles during growth on 1,2-propanediol–CN-B_{12} minimal medium (6). Based on electron microscopy studies and analogy with carboxysomes, we proposed that these organelles consisted of a proteinaceous shell that encased coenzyme B_{12}-dependent diol dehydratase and perhaps other enzymes (6). Here we present genetic and biochemical evidence that PduA is a shell protein of the polyhedral organelles of *S. enterica*. Immunolabeling experiments showed that the PduA protein localized to the periphery of the organelles (Fig. 3C). Strains having nonpolar *pduA* mutations were unable to synthesize organelles (Fig. 3D), and expression of the PduA protein from a plasmid vector resulted in aberrantly shaped organelles at higher expression levels (Fig. 4). Thus, three independent lines of evidence indicate that the PduA protein is a component of the shell of the polyhedral organelles of *S. enterica*.

The results reported here are also consistent with the localization of diol dehydratase within the shell of the organelle. A $\Delta pduA$ strain (organelle negative) grew faster than the wild type at limiting B_{12} concentrations, suggesting that the organelles act as barriers to B_{12}. In strains with *pduA* mutations, diol dehydratase was no longer associated with the organelles but was found in large inclusion bodies located at the poles of the cell (Fig. 3A and B). Prior immunogold labeling studies showed that diol dehydratase localized primarily to the interior of the organelles (6). Hence, several findings indicate that diol dehydratase localizes within the shell of the organelles. However, the possibility that diol dehydratase is also a component of the organelle shell cannot be excluded at this time.

The shell of the *S. enterica* polyhedral organelles is likely to be composed of several proteins in addition to the PduA protein. There are four carboxysome shell protein homologues encoded within the *pdu* operon, PduAJKT, and previous studies showed that polar insertion mutations downstream of *pduH* caused the formation of aberrant organelles (6). Furthermore, the shells of carboxysomes are known to be composed of multiple proteins (34, 35), and the occurrence of multiple shell gene homologues within an operon has been observed previously in *Halothiobacillus* (2, 3), *Synechococcus* (17, 18, 22, 24), and *Synechocystis* (39).

The function of the *Salmonella* organelles is currently unknown, but it seems likely that the shells of these organelles act as permeability barriers. Some models propose that carboxysomes concentrate CO_2 for RuBisCO, which is encased within the shell of the organelle (15, 25, 34, 35). Similarly, the *S. enterica* organelles might be used to concentrate 1,2-propanediol (the substrate), or Ado-B_{12} (the required cofactor) for diol dehydratase. However, the growth studies reported

here showed that a *pduA* mutant unable to form organelles grew similarly to or better than the wild type on lower concentrations of 1,2-propanediol and CN-B$_{12}$ (a precursor of Ado-B$_{12}$). These results indicated that the *S. enterica* organelles are not involved in the concentration of 1,2-propanediol or Ado-B$_{12}$.

Previously it was suggested that the polyhedral organelles of *S. enterica* were used to sequester toxic aldehydes (36). *S. enterica* forms polyhedral organelles during B$_{12}$-dependent growth on both 1,2-propanediol and ethanolamine, and aldehyde intermediates are a common feature of both degradative pathways (13, 28). The studies reported here support a model in which the polyhedral organelles of *S. enterica* function to minimize aldehyde toxicity. The finding that Δ*pduA* mutants undergo a period of interrupted growth at higher 1,2-propanediol concentrations but not at lower 1,2-propanediol concentrations is consistent with formation of a toxic compound derived from 1,2-propanediol. In the first step of 1,2-propanediol degradation, coenzyme B$_{12}$-dependent diol dehydratase catalyzes the formation of propionaldehyde from 1,2-propanediol. Presumably, higher concentrations of 1,2-propanediol would result in a higher rate of propionaldehyde formation and in greater toxicity. In addition, *pduA* mutants showed interrupted growth on 1,2-propanediol minimal medium supplemented with higher levels of CN-B$_{12}$ but not on similar medium supplemented with lower levels of CN-B$_{12}$. *S. enterica* converts CN-B$_{12}$ to Ado-B$_{12}$, the required cofactor for diol dehydratase. Hence, at higher CN-B12 concentrations, it is expected that propionaldehyde would be formed faster and greater toxicity would result. Following the period of interrupted growth, mutants unable to form organelles resumed growth at a rate similar to that of the wild-type strain. Presumably, growth resumed after the induction of appropriate stress-response genes.

Results also showed that mutant strains unable to produce polyhedral organelles grew substantially faster than the wild-type strain on 1,2-propanediol minimal medium supplemented with lower levels of CN-B$_{12}$. This result indicated that the *S. enterica* organelles present a barrier to Ado-B$_{12}$. Although this would result in slower growth at lower CN-B$_{12}$ (Ado-B$_{12}$) concentrations, it could help minimize propionaldehyde toxicity at higher CN-B$_{12}$ concentrations. Interestingly, this suggests a model in which the polyhedral organelles of *S. enterica* function to minimize aldehyde toxicity by moderating the rate of propionaldehyde production through the control of Ado-B$_{12}$ availability.

ACKNOWLEDGMENTS

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