

POLYHEDRAL ORGANELLES INVOLVED IN THE B₁₂-DEPENDENT
METABOLISM OF 1,2-PROPANEDIOL IN *Salmonella enterica* SEROVAR
TYPHIMURIUM LT2

By

GREGORY DALE HAVEMANN

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA
2003

Copyright 2003

by

Gregory Dale Havemann

I dedicate this work to my parents and my sister for their endless support and patience
and to Stephanie for putting up with me through all of this.

ACKNOWLEDGMENTS

I would like to thank my mentor and guide over the years, Dr. Thomas Bobik; his love of science and dedication were an inspiration to me in times of difficulty.

Special thanks go to Dr. Henry Aldrich and the members of the electron microscopy lab, Donna Williams and Lorraine McDowell, all of whom taught me the ins and outs of electron microscopy and endured my constant barrage of questions.

I would like to thank the rest of my committee: Dr. K.T. Shanmugam, whose genetics lab first turned me on to the interesting and challenging field of bacterial genetics; Dr. Madeline Rasche, whose advice greatly facilitated my work and whose enthusiasm for science is contagious; and Dr. Paul Gulig, whose knowledge of replacement vectors made mutant construction a less difficult task.

I would like to acknowledge Tetsuo Toraya for providing the anti-diol dehydratase antiserum, which was crucial for western blots and immunolabeling.

I am grateful to the personnel of the Protein Chemistry Core at the University of Florida, Interdisciplinary Center for Biotechnology Research who performed the 2D-PAGE separations, the N-terminal sequencing, and the MALDI-TOF analysis. Specifically I would like to thank Marjorie Chow, Charity Hartmann, Alexia Lunberg, Scott McMillen, Scott McClung, and Li Zhang who all performed a portion of the work.

My thanks also go to Nicole Leal, Celeste Johnson, and Angel Sampson. Nicole provided the anti-PduP antiserum and performed propionaldehyde dehydrogenase assays, Celeste provided the anti-PduO antiserum and performed adenosyltransferase assays, and

Angel provided me with strain BE39. I would also like to thank all of the members of the Bobik lab for making the lab a pleasant working environment.

The text of Chapter 3 in this dissertation, in part or in full, is a reprint of the material as it appears in the *Journal of Bacteriology* (volume 184, pp. 1253-1261) and Figure 2-8 is from *Journal of Bacteriology* (volume 181, pp. 5973).

TABLE OF CONTENTS

	<u>Page</u>
ACKNOWLEDGMENTS	iv
LIST OF TABLES.....	x
LIST OF FIGURES	xi
ABSTRACT.....	xiii
CHAPTER	
1 INTRODUCTION	1
Origins of B ₁₂	2
Distribution of B ₁₂	2
Structure of B ₁₂	2
Biosynthesis of B ₁₂	5
Transport of B ₁₂	5
B ₁₂ -Dependent Reactions.....	6
B ₁₂ -Dependent Reactions in Enteric Bacteria	7
Propanediol dehydratase	7
Ethanolamine ammonia lyase.....	9
Glycerol dehydratase.....	9
Methionine synthetase.....	9
Epoxyqueuosine reductase	10
Other B ₁₂ -Dependent Reactions	10
Acetyl CoA synthesis	10
Methyl transfer in the methane-producing Archaea.....	11
Ribonucleotide reductases.....	11
Degradation of recalcitrant compounds	11
Methylmalonyl-CoA mutase	12
<i>S. enterica</i> as a Model Organism for the Study of B ₁₂ Physiology and Metabolism .12	
B ₁₂ -Dependent PD Degradation in <i>S. enterica</i>	13
Degradation of Propanediol.....	13
1,2-propanediol	13
The <i>pdu</i> locus	13
Regulation	15
The pathway of PD degradation in <i>S. enterica</i>	17
Homologues of Carboxysomal Proteins	19

The Carboxysome.....	19
Components of the Carboxysome	20
Function of the Carboxysome	20
Research Overview	21
2 ANALYSES OF POLYHEDRAL ORGANELLE FORMATION IN <i>SALMONELLA ENTERICA</i> SEROVAR TYPHIMURIUM LT2	24
Introduction.....	24
Materials and Methods	26
Chemicals and Reagents.....	26
Bacterial Strains, Media, and Growth Conditions.....	27
TPOP Mutagenesis	28
Localization of TPOP Insertions	28
Genetic Techniques	29
Cloning of the <i>pduB</i> Gene for Complementation Studies	30
Complementation Studies.....	30
Electron Microscopy	31
Construction of In-Frame <i>pduCDE</i> Deletion	32
Results.....	34
Polyhedral Organelles are Formed in Cells Grown on Poor Carbon Sources in the Presence of PD	34
Time Course of Organelle Formation.....	37
Screening of TPOP Mutants	39
Effect of TPOP Insertions on the Formation of Polyhedral Organelles.....	39
Complementation of the Polyhedra Organelle-Negative Phenotype of <i>pduA</i> and <i>pduB</i> TPOP Insertion Mutants	40
The B ₁₂ -Dependent Diol Dehydratase is not Required for the Formation of the Polyhedral Organelles.....	41
Localization of Plasmid Encoded Diol Dehydratase in a Δ <i>pduCDE</i> Mutant.....	44
Genes Downstream of Diol Dehydratase are Required for the Formation of Polyhedral Organelles.....	44
Discussion.....	49
3 PDUA IS A SHELL PROTEIN OF POLYHEDRAL ORGANELLES INVOLVED IN THE B₁₂-DEPENDENT DEGRADATION OF 1,2-PROPANEDIOL IN <i>SALMONELLA ENTERICA</i> SEROVAR TYPHIMURIUM LT2.....	54
Introduction.....	54
Materials and Methods	56
Chemicals and Reagents.....	56
Bacterial Strains, Media, and Growth Conditions.....	57
General Protein and Molecular Methods.....	57
P22 Transduction and Transposon Mutagenesis of the <i>pdu</i> Region	58
Localization of TPOP1 Insertions	58
Growth Curves.....	58
Cloning of <i>pduA</i> for High-Level Expression.....	60

Cloning of <i>pduJ</i> for High-Level Expression	60
Purification of the PduA Protein	61
Antiserum Preparation.....	61
Western Blots	62
Electron Microscopy	63
Construction of a Nonpolar <i>pduA</i> Deletion	64
DNA Sequencing and Analysis.....	64
Results.....	65
Purification of Recombinant His ₆ -PduA Protein.....	65
Preparation of PduA-Specific Antiserum	68
Localization of PduA by Immunoelectron Microscopy	70
Localization of Diol Dehydratase in a <i>pduA</i> Mutant.....	70
The <i>pduA</i> Gene is Required for the Formation of Polyhedral Organelles	72
Co-Localization of the PduA Protein and Diol Dehydratase.	72
Complementation of the Polyhedral Organelle Minus Phenotype of a <i>ΔpduA</i> Mutant Strain	74
Strains with <i>pduA</i> Mutations Show a Period of "Interrupted" Growth when Cultured on PD/CN-B ₁₂ Minimal Medium.....	76
CO ₂ Supplementation Does not Correct the "Interrupted" Growth Phenotype of a <i>pduA</i> Mutant Strain.....	78
Effects of PD Concentration on the Growth Rates of Wild Type and <i>pduA</i> Mutant Strains.....	78
Effects of CN-B ₁₂ Concentration on the Growth Rates of Wild Type and <i>pduA</i> Null Mutant Strains.....	83
Discussion.....	85
4 PURIFICATION AND IDENTIFICATION OF THE MAJOR PROTEINS OF POLYHEDRAL ORGANELLES INVOLVED IN COENZYME B₁₂-DEPENDENT DEGRADATION OF 1,2-PROPANEDIOL IN <i>SALMONELLA ENTERICA</i> SEROVAR TYPHIMURIUM LT2	89
Introduction.....	89
Materials and Methods	92
Chemicals and Reagents.....	92
Organelle Purification	92
General Protein and Molecular Methods.....	94
Electron Microscopy	94
Western Blots	94
Enzyme Assays.....	94
Glycoprotein Staining.....	95
N-terminal Sequencing.....	95
Matrix Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry (MALDI-TOF MS).....	96
Peptide Mass Fingerprinting.....	97
Densitometry Analysis	98
Results.....	98
Purification of Polyhedral Organelles	98

Electron Microscopy	100
SDS-PAGE Analysis	102
Diol Dehydratase Activity of the Polyhedral Organelles	102
Other Activities of the Polyhedral Organelles.....	104
Western Blot Analysis of Purified Polyhedral Organelles	105
Glycoprotein Staining of Organelle Proteins	107
Two-Dimensional Electrophoretic Analysis of Purified Polyhedral Organelles	108
N-Terminal Sequencing.....	108
MALDI-TOF Analysis	110
Unidentified Protein of the Purified Polyhedral Organelles.....	118
Densitometry, Molecular Mass, and pI Values of Polyhedral Organelle Proteins Separated by Two-Dimensional Electrophoresis.....	115
Discussion.....	119
5 CONCLUSIONS	125
Conditions Required for the Formation of the Polyhedral Organelles	125
Genes Involved in the Formation of the Polyhedral Organelles.....	126
PduA is a Shell Protein Required for the Formation of the Polyhedral Organelles .	127
Purification of the Polyhedral Organelles.....	128
The Polyhedral Organelles are Composed of at Least 14 Proteins	129
A Model for the Polyhedral Organelles of <i>S. enterica</i>	130
Future Experimentation	131
LIST OF REFERENCES.....	134
BIOGRAPHICAL SKETCH	148

LIST OF TABLES

<u>Table</u>	<u>page</u>
2-1. Bacterial strains	29
2-2. Conditions under which polyhedral organelles are formed.....	35
3-1. Bacterial strains	59
4-1. Diol dehydratase activity during organelle purification	103
4-2. Propionaldehyde dehydrogenase activity during organelle purification	104
4-3. Organelle proteins identified using N-terminal sequencing.....	110
4-4. Organelle proteins identified by peptide mass fingerprinting	112
4-5. Molecular mass, pI, and densitometry analyses	117

LIST OF FIGURES

<u>Figure</u>	<u>page</u>
1-1. Structure of cobalamins	4
1-2. B ₁₂ -dependent pathways known in enteric bacteria	8
1-3. The <i>pdu</i> operon.....	14
1-4. Regulation of the <i>pdu/cob</i> regulon	16
1-5. Putative pathway of aerobic PD degradation in <i>S. enterica</i>	18
2-1. Construction of plasmid pGH13 used in generating the <i>pduCDE</i> deletion in strain BE87.....	33
2-2. Electron micrographs of <i>S. enterica</i> grown under different conditions	36
2-3. Aerobic growth of <i>S. enterica</i> on PD minimal medium	38
2-4. Complementation of a <i>pduA</i> ::TPOP insertion mutant.....	42
2-5. Complementation of a <i>pduB</i> ::TPOP insertion mutant.....	43
2-6. Electron micrograph of a <i>ΔpduCDE</i> mutant grown on succinate minimal medium in the presence of PD	45
2-7. Packaging of diol dehydratase	47
2-8. Electron micrograph of the aberrant polyhedra formed by <i>S. enterica pdu</i> mutant RT818 (<i>pdu-8::MudJ</i>)	48
3-1. Overexpression and purification of the His ₆ -PduA protein.....	66
3-2. Overexpression of the His ₆ -PduA protein in <i>E. coli</i> strain BE230.....	67
3-3. Western analysis with untreated and absorbed anti-PduA polyclonal antiserum preparations	69
3-4. Localization of diol dehydratase and the PduA protein by immunoelectron microscopy	71

3-5. Co-localization of diol dehydratase and the PduA protein.....	73
3-6. Complementation of a <i>ΔpduA</i> mutation for formation of polyhedral organelles.....	75
3-7. Growth of the wild type strain and a <i>pduA</i> mutant on PD/CN-B ₁₂ minimal medium	77
3-8. The effect of high-CO ₂ on growth of the wild type strain and a <i>pduA</i> mutant on PD/CN-B ₁₂ minimal medium	79
3-9. The effect of high-CO ₂ and VILT supplementation on the growth of strain BE182 (<i>ΔpduA</i>) and the wild type strain.....	80
3-10. The effect of various PD concentrations on the growth of strain BE182 (<i>ΔpduA</i>) and the wild type strain	81
3-11. The effects of various CN-B ₁₂ concentrations on the growth of strain BE182 (<i>ΔpduA</i>) and the wild type strain (<i>S. enterica</i>)	84
4-1. Electron micrographs of polyhedral organelles purified from <i>S. enterica</i>	99
4-2. Purification of the polyhedral organelles of <i>S. enterica</i>	101
4-3. Western analysis of purified polyhedral organelles	106
4-4. Two-dimensional electrophoresis of purified polyhedral organelles of <i>S. enterica</i> used for N-terminal sequencing	109
4-5. Two-dimensional electrophoresis of purified polyhedral organelles of <i>S. enterica</i> used for MALDI-TOF analysis	111
4-6. MALDI-TOF MS peptide map of spot 11	113
4-7. Densitometry of the polyhedral organelle proteins	116
4-8. A model of the <i>pdu</i> organelle	122

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

POLYHEDRAL ORGANELLES INVOLVED IN THE B₁₂-DEPENDEDENT
METABOLISM OF 1,2-PROPANEDIOL IN *Salmonella enterica* SEROVAR
TYPHIMURIUM LT2

By

Gregory Dale Havemann

May 2003

Chair: Thomas A. Bobik

Major Department: Microbiology and Cell Science

In an effort to better understand salmonella physiology and coenzyme B₁₂-dependent processes, *Salmonella enterica* has been used as a model to study coenzyme B₁₂-dependent degradation of 1,2-propanediol. One of the most surprising findings was the observation of polyhedral organelles in *S. enterica* cells grown on propanediol. Although these organelles appeared similar to carboxysomes, which are involved in CO₂-fixation in autotrophic bacteria, a similar function in *S. enterica* was unlikely since this organism is not an autotroph and does not fix CO₂. This dissertation investigates the structure and function of these unusual bacterial organelles.

The conditions required for organelle formation were defined, and a time course of organelle formation revealed that their synthesis correlated to a metabolic shift to growth on propanediol. In addition, two genes, *pduA* and *pduB*, were shown to be involved in organelle formation. To further investigate the role of the PduA protein in polyhedral organelle formation, the *pduA* gene was cloned and overexpressed. The protein product

was then used to generate polyclonal antiserum. Immunolabeling with this antiserum demonstrated that the PduA protein localized to the organelle periphery, suggesting it was a component of the shell. Additional studies demonstrated that *S. enterica pduA* null mutants did not make polyhedral organelles, and when grown on propanediol minimal medium they exhibited a period of arrested growth. Subsequent physiological tests suggested that the organelles might function as a B₁₂ barrier, and the arrested growth observed in organelle mutants may result from increased production of a toxic intermediate.

In order to determine the constituent proteins of the polyhedral organelles, a purification scheme was developed to obtain stable, homogenous preparations. After purification, preparations were separated using one and two-dimensional gel electrophoresis. The major proteins of the organelle were identified using peptide mass fingerprinting, N-terminal sequencing, and immunoblotting. A total of 14 proteins was identified, including four enzymes: coenzyme B₁₂-dependent-diol dehydratase, CoA-dependent propionaldehyde dehydrogenase, adenosyltransferase, and a putative two-component diol dehydratase-reactivating factor. Based on these findings, a model is proposed wherein the polyhedral organelles serve to prevent cellular toxicity by channeling and sequestering propionaldehyde as well as by moderating the rate of its production.

LIST OF ABBREVIATIONS

A	adenine
ADP	adenosine diphosphate
Ado-B ₁₂	adenosyl-B ₁₂ or adenosylcobalamin
AIM	aldehyde indicator medium
Amp	ampicillin
ATP	adenosine triphosphate
B-PER II [®]	bacterial protein extraction reagent-II
BSA	bovine serum albumin
C	cytosine
°C	centigrade
cAMP	cyclic adenosine monophosphate
CH ₃ -B ₁₂	methyl-B ₁₂ or methylcobalamin
Cam	chloramphenicol
cm	centimeter
CN-B ₁₂	cyano-B ₁₂ , vitamin B ₁₂ or cyanocobalamin
<i>cob</i>	cobalamin
ddH ₂ O	distilled deionized water
dH ₂ O	deionized water
DMB	dimethylbenzimidazole
DNA	deoxyribonucleic acid

DNase	deoxyribonuclease
DTT	dithiothreitol
<i>E. coli</i>	<i>Esherichia coli</i>
EDTA	ethylenediamine tetraacetate
EM	electron microscopy
G	guanosine
HO-B ₁₂	hydroxy-B ₁₂ , hydroxycobalamin
hr	hour
IPG	immobilized pH gradient
IPTG	isopropyl-β-D-thiogalactopyranoside
Kan	kanamycin
kDa	kiloDaltons
λ	wavelength (nm)
LB	Luria-Bertani medium
M	molar concentration
MALDI-TOF	matrix assisted laser desorption/ionization time of flight
MeOH	methanol
MES	morpholino ethanesulfonic acid
min	minutes
mM	millimolar
MOPS	morpholino propanesulfonic acid
MS	mass spectrometry
NaCl	sodium chloride

NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide reduced form
NCE	No-carbon-E-medium
nm	nanometer
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD	1,2-propanediol
<i>Pfu</i>	<i>Pyrococcus furiosus</i>
RPM	revolutions per minute
SDS	sodium-dodecyl-sulfate
<i>S. enterica</i>	<i>Salmonella enterica</i> serovar typhimurium LT2
T	thymidine
<i>Taq</i>	<i>Thermus aquaticus</i>
tet	tetracycline
THF	tetrahydrofolate
Tris	tris (hydroxymethyl) aminomethane
tRNA	transfer ribonucleic acid
V	volt

CHAPTER 1 INTRODUCTION

In 1926, Minot and Murphy discovered that a diet containing high amounts of liver would prevent death in individuals suffering from pernicious anemia (Minot and Murphy 1926). It was not until 1948 that two independent research teams led by K. Folkers and E.L. Smith isolated the active component of this liver diet and agreed to name it vitamin B₁₂ (Rickes et al. 1948, Smith and Parker 1948). In 1950, these research teams joined two additional teams (led by Sir Alexander Todd and D. C. Hodgkin), and solved the chemical structure of B₁₂ in the ensuing 6 years (Hodgkin et al. 1955). Soon after, Lenhert and Hodgkin solved the structure of a newly discovered coenzymatic form of B₁₂, adenosyl-B₁₂ (Ado-B₁₂) (Lenhert and Hodgkin 1961). In the next year yet another coenzymatic form of B₁₂ was discovered (methyl-B₁₂), which was found to be a cofactor for methionine synthase (Guest et al. 1962a, Guest et al. 1962b). By 1972, more than ten coenzyme B₁₂-dependent enzymes or enzyme systems had been discovered and characterized (Schneider 1987). Presently, at least fifteen coenzyme B₁₂-dependent enzymes are known (Schneider 1987, Roth et al. 1996).

Vitamin B₁₂ is required for methionine synthesis and propionate catabolism in higher animals. Among prokaryotes, it appears to play an important role in a number of metabolic pathways that have ecological and industrial significance including several amino acid and carbohydrate fermentations, methane production, carbon dioxide fixation via the acetyl-CoA pathway (Wood et al. 1986, Ragsdale 1991, Stupperich 1993, Ferry 1995), acetogenesis (Ljungdahl 1982, Ragsdale 1991), ribonucleotide reduction, (Blakley

and Barker 1964, Blakley 1965, Reichard 1993, Booker et al. 1994), methionine biosynthesis and the anaerobic catabolism of recalcitrant polymers (Frings et al. 1992, Frings and Schink 1994, Frings et al. 1994).

Origins of B₁₂

Many have postulated that B₁₂ was present prebiotically and may have played an important catalytic role in the “RNA world” (Benner et al. 1989). The porphyrin molecule, a precursor to the central corrin ring of B₁₂, has been synthesized in “primitive earth” experiments (Hodgson and Ponnamperuma 1968), and Eschenmoser (1988) has commented on how the B₁₂ structure could have been formed using energetically favorable reactions. Roth and colleagues (1996) proposed that the original function of B₁₂ was to balance redox reactions by providing an internal electron sink. Later it evolved to allow the use of inorganic electron acceptors. Finally with the development of an oxygen atmosphere on Earth, B₁₂ assumed roles in aerobic metabolism in the form of heme and chlorophyll.

Distribution of B₁₂

Among the kingdoms of life, B₁₂ is unevenly distributed. Although only microorganisms are capable of synthesizing B₁₂ de novo, both mammals and protists require this cofactor for their metabolism. Plants and fungi, with few exceptions, appear to neither synthesize nor require this cofactor for their metabolism (Duda et al. 1967) and instead may use S-adenosyl methionine as a source of the 5'-deoxyadenosyl radical required in carbon skeleton rearrangements (Ollagnier et al. 1998).

Structure of B₁₂

With a molecular weight of 1350 Da, vitamin B₁₂ (CN-B₁₂) or cyano-cobalamin is the largest cofactor known to man. It is composed of four parts: a central ring, an

aminopropanol side chain, a lower ligand (α), and an upper ligand (β) (Figure 1-1). The central ring is composed of four pyrrole molecules that are joined to form a macrocyclic ring similar to the uroporphyrinogen (UroIII) molecule, a precursor of this ring structure as well as those of heme, chlorophyll, and siroheme. Like UroIII, the macrocyclic ring of vitamin B₁₂ is a tetrapyrrole, however it differs in that it lacks a carbon bridge between the porphyrin A and D rings and has a different ring oxidation state. The central ring of vitamin B₁₂ is referred to as “corrin” and was named so by its founders because it is the “core” molecule of B₁₂. After the addition of the central cobalt atom and decoration with methyl, acetamide, and propionamide groups this structure becomes known as cobyrinic acid. Adding an aminopropanol side chain forms cobinamide and further modification by addition of a phosphoribosyl group to the aminopropanol side chain results in cobamide. Dimethylbenzimidazole (DMB) is the α -ligand in vitamin B₁₂. It is attached to the ring covalently by the aminopropanol side chain and is coordinated to the central cobalt atom. Collectively, corrinoids of this type are known as cobalamins. Cobalamin is prepared commercially as cyanocobalamin (CN-B₁₂) a form that is uncommon in nature but frequently used as a supplement for humans and bacterial mutants. Other cobinamide α ligands have been discovered in anaerobic bacteria and Archaea but studies conducted thus far have not elucidated the significance of these alternative α ligands (Brown et al. 1955, Fantes and O'Callaghan 1955, Ford et al. 1955, Stupperich et al. 1987, Stupperich et al. 1990) and at present they appear isofunctional to DMB (Stupperich et al. 1990). The β ligand of cobalamins can be a number of different chemical groups but four of the more common are: hydroxy, cyano, methyl and adenosyl. It is the latter two groups

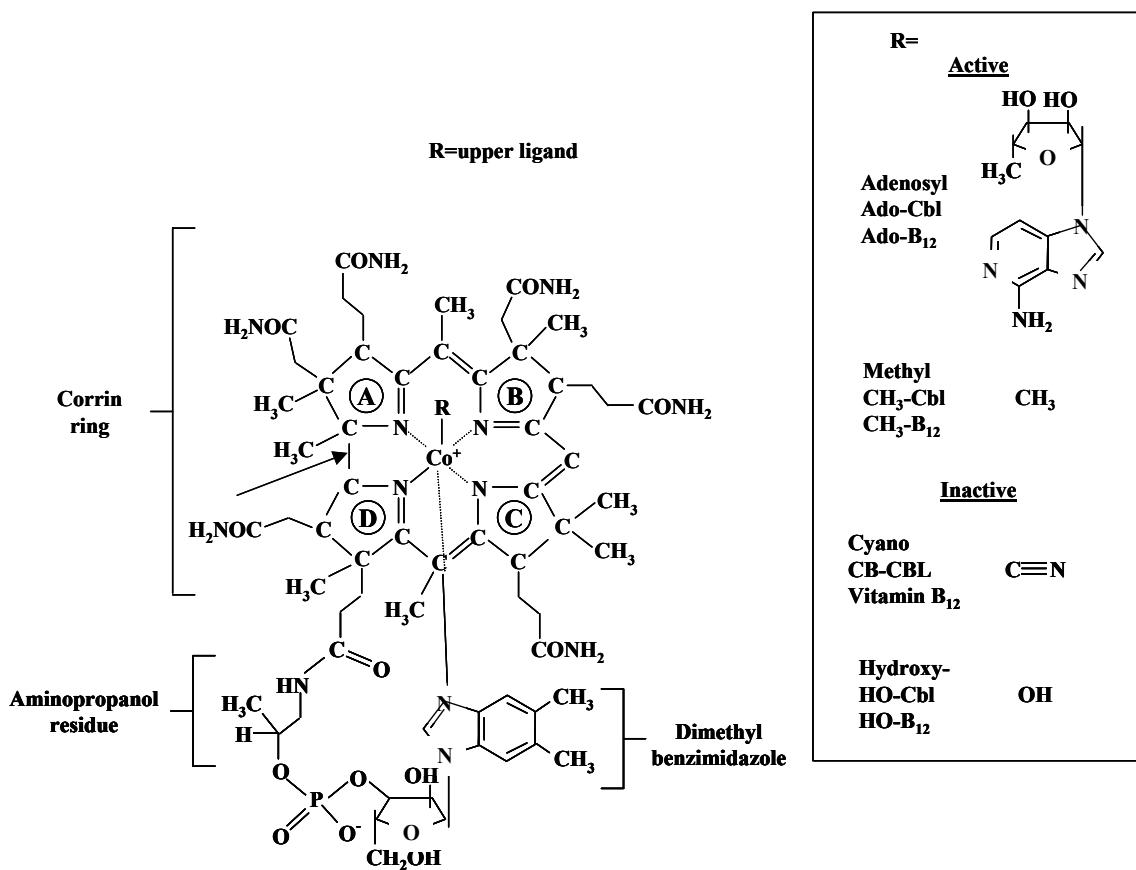


Figure 1-1. Structure of cobalamins. The major parts of the cofactor are indicated: corrin ring, dimethylbenzimidazole, aminopropanol residue, and the upper ligand. The absence of the carbon bridge between the A and D porphyrin rings is denoted by the arrow. The upper ligand can be any of a number of chemical groups but the four most common groups are shown in the legend.

however, that are components of the active coenzyme forms: adenosylcobalamin (Ado-B₁₂) and methylcobalamin (CH₃-B₁₂).

Biosynthesis of B₁₂

In *S. enterica* the genes required for the biosynthesis of B₁₂ or cobalamin are located at the *cob* operon. This operon is coregulated with genes required for the degradation of propanediol (PD), which are located in the adjacent and divergently transcribed *pdu* operon, indicating that degradation of PD may be the primary reason for de novo synthesis of B₁₂ in *S. enterica*. Several pathways for B₁₂ synthesis exist in microorganisms, some occur aerobically, and others can occur only in the absence of oxygen. In *Klebsiella aerogenes*, B₁₂ is made both aerobically and anaerobically, making it an ideal organism to study the differences in these pathways. In *S. enterica* however, B₁₂ is made only under anaerobic conditions.

Transport of B₁₂

In addition to synthesizing B₁₂ de novo, *S. enterica* can scavenge corrinoids from its environment for conversion into B₁₂. The transport of B₁₂ in *Salmonella* is rather problematic since B₁₂ is present only in minute quantities in the environment and is also a large molecule that exceeds the limit for entry via outer membrane porins. The mechanism of B₁₂ transport has been extensively studied in *E. coli* and consists of two separate transport systems, one for the outer membrane and a second for the inner membrane (Bradbeer 1991). Transport across the outer membrane entails binding to the BtuB protein which has a high affinity for vitamin B₁₂ and its derivatives (Taylor et al. 1972, White et al. 1973, Bradbeer et al. 1978, Kenley et al. 1978). Once bound to BtuB, the B₁₂-BtuB complex interacts with the TonB protein which provides energy derived from a proton-motive force to drive a structural alteration of the BtuB protein resulting in

translocation of B₁₂ across the outer membrane (Bradbeer and Woodrow 1976, Bradbeer 1993). Once inside the periplasm, it is thought that the BtuF protein binds B₁₂ (White et al. 1973) which then transports it to the BtuCD complex on the inner membrane where translocation of B₁₂ into the cytosol is driven by the hydrolysis of ATP (Kadner and Liggins 1973, Bassford Jr. and Kadner 1977, DeVeaux and Kadner 1985, DeVeaux et al. 1986).

B₁₂-Dependent Reactions

The vitamin B₁₂ coenzymes, Ado-B₁₂ and CH₃-B₁₂, are cofactors for at least 15 different enzymes. Ado-B₁₂ is used as a cofactor for enzymes involved in intramolecular rearrangement reactions. In the enteric bacteria, examples of such reactions can be seen in the fermentation of small molecules such as 1,2-propanediol (PD), ethanolamine, and glycerol. In other prokaryotes, Ado-B₁₂ is used in the fermentation of amino acids such as glutamate, lysine, leucine, and ornithine (Stroinski 1987). Among higher organisms, Ado-B₁₂ is used in the metabolism of propionyl-CoA. The key step in each of these reactions is the formation of an adenosyl radical on the Ado-B₁₂ molecule, which results from the homolytic cleavage of the carbon-cobalt bond (Stroinski 1987). Ado-B₁₂ then abstracts a proton from the substrate forming an unstable radical that rearranges forming the product. Methylcobalamin (CH₃-B₁₂), on the other hand is the cofactor for methyltransferases (Schneider and Stroinski 1987b), which are involved in methionine synthesis in both higher organisms and bacteria, in methanogenesis in the methanogens, and in acetyl-CoA synthesis in anaerobic bacteria. Reactions utilizing this cofactor do not involve homolytic cleavage of the carbon-cobalt bond and instead proceed using ionic interactions (Banerjee and Matthews 1990, Drennan et al. 1994, Ludwig et al. 1996, Ludwig and Matthews 1997).

B₁₂-Dependent Reactions in Enteric Bacteria

The enzymes listed below are found in one or more enteric bacteria, and all of them except glycerol dehydratase are found in *S. enterica*. The reactions catalyzed by each of these enzymes are diagrammed in [Figure 1-2](#). A more detailed description of their role in metabolism is reserved for later.

Propanediol dehydratase

Diol dehydratase is one of the best-studied Ado-B₁₂-dependent enzymes. Research on this enzyme, which is found in nearly all enteric bacteria tested except *E. coli* (Toraya et al. 1979, Lawrence and Roth 1996), has provided a wealth of information on the mechanisms of Ado-B₁₂-dependent reactions (Toraya 2002). Ado-B₁₂-dependent diol dehydratase is the initiating enzyme in the degradation of PD where it catalyzes the conversion of PD to propionaldehyde (Abeles and Lee Jr. 1961). All enterics possessing this enzyme are capable of aerobically respiring PD, which provides both an energy source via substrate level phosphorylation and a three-carbon compound that can enter central metabolism via the 2-methylcitric acid pathway (Horswill and Escalante-Semerena 1997, Tsang et al. 1998). Only some of the enterics ferment PD by first converting it to propionaldehyde and then subsequently dismutating it to propionate and propanol ([Figure 1-2](#)). In *Salmonella* this process produces a source of energy from substrate-level phosphorylation but no source of carbon since propanol is excreted in order to get rid of excess reducing equivalents (Price-Carter et al. 2001). Recently it has been found that the use of tetrathionate as a terminal electron acceptor allows PD to be used as both an energy and carbon source under anaerobic conditions (Price-Carter et al. 2001).

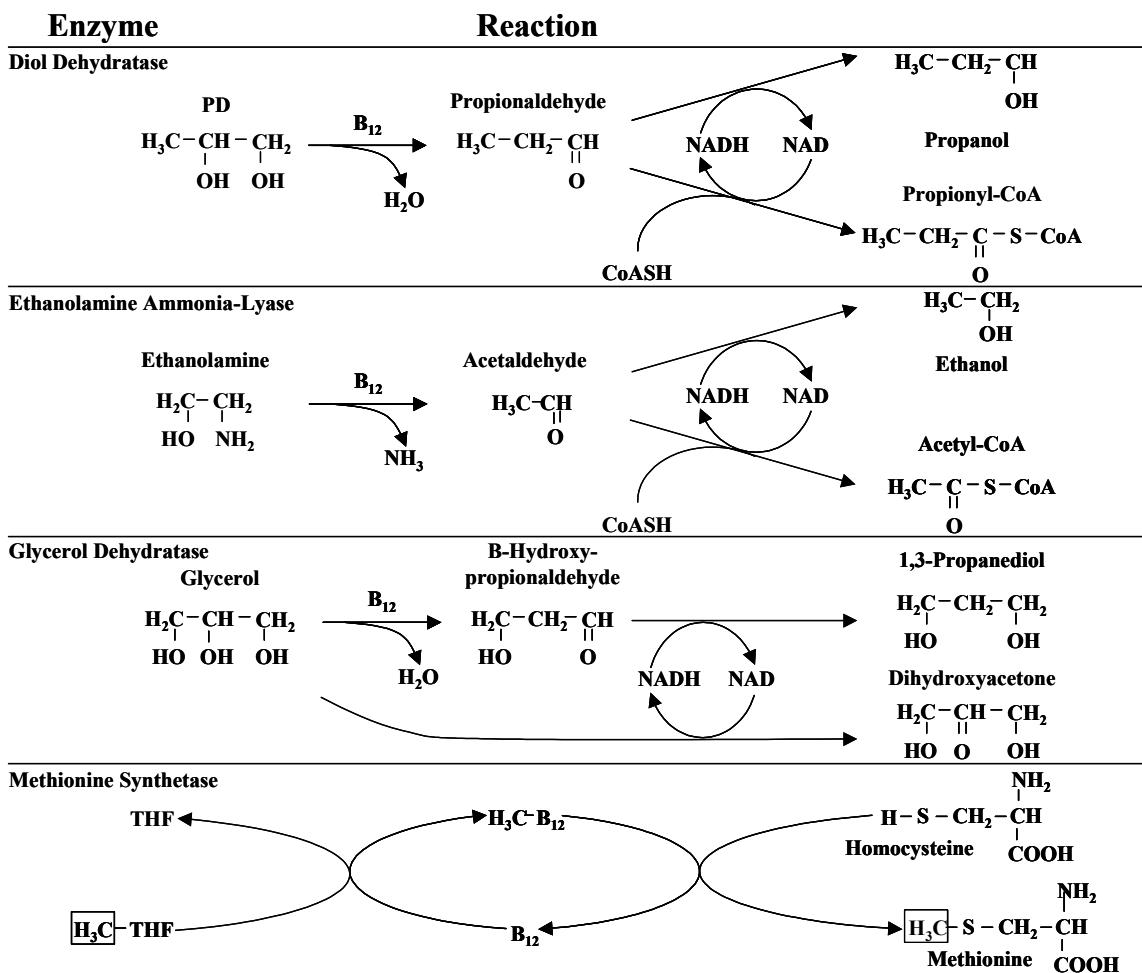


Figure 1-2. B_{12} -dependent pathways known in enteric bacteria. Only the first reaction for each pathway utilizes B_{12} as a cofactor. THF, tetrahydrofolate.

Ethanolamine ammonia lyase

Ado-B₁₂-dependent ethanolamine ammonia lyase is used for ethanolamine degradation (Bradbeer 1965, Scarlett and Turner 1976). The pathway for ethanolamine degradation is analogous to that for PD: ethanolamine is converted to acetaldehyde with the concomitant loss of ammonia and is further metabolized to both ethanol and acetic acid. This process in addition to providing an energy and carbon source, also provides a source of nitrogen. The induction of the *eut* operon requires both ethanolamine and B₁₂, and unlike PD ethanolamine does not induce the de novo synthesis of B₁₂ (Rondon and Escalante-Semerena 1992, Chen et al. 1995, Bobik et al. 1997) Ethanolamine is encountered frequently in nature as a component of the lipids, phosphatidyl ethanolamine and phosphatidyl choline.

Glycerol dehydratase

This enzyme converts glycerol to β-hydroxypropionaldehyde, which can subsequently be reduced to 1,3-propanediol, a commercially important compound used in the manufacturing of carpet backing (Homann et al. 1990, Witt et al. 1994). This reaction provides a means to regenerate reducing equivalents produced by glycerol dehydrogenase. Glycerol is commonly found as the backbone of lipids.

Methionine synthetase

The final step in the synthesis of methionine in both humans and many bacteria requires CH₃-B₁₂-dependent methionine synthetase, an enzyme that serves to transfer methyl groups from methyltetrahydrofolate to homocysteine. Methionine synthase is one of the better studied B₁₂-dependent enzymes partly because defects in this enzyme result in hyperhomocysteinuria, a possible risk factor in heart disease (Refsum et al. 1998, Siri et al. 1998). Both *Salmonella* spp. and *E. coli* present ideal models to study this enzyme

since they possess two different methionine synthetase enzymes: the B₁₂-dependent Meth and MetE, which does not require B₁₂ as a cofactor. The Meth enzyme is used preferentially when B₁₂ is available and MetE is induced in response to accumulated homocysteine in *metH* mutants (Wu et al. 1992).

Epoxyqueuosine reductase

Epoxyqueuosine reductase performs the final step in the formation of the hypermodified tRNA base, queuosine, found in the first anticodon position of tRNA^{asn}, tRNA^{asp}, tRNA^{his}, and tRNA^{tyr}. This modified base has been demonstrated to be nonessential for bacterial growth under laboratory conditions (Noguchi et al. 1982). The final reaction in its synthesis has been reported to require B₁₂ (Frey et al. 1988). However, in *E. coli*, which lacks the genes for de novo B₁₂ synthesis and which was grown in medium lacking suitable corrinoid precursors for B₁₂ production, epoxyqueuosine could still be made. Hence, it has been suggested that the reaction does not require B₁₂ but may be stimulated by it indirectly (Roth et al. 1996).

Other B₁₂-Dependent Reactions

Acetyl CoA synthesis

A methyl-corrinoid/iron sulfur protein serves to transfer methyl groups in many anaerobic bacteria that utilize the Wood/Ljungdahl pathway to synthesize acetyl-CoA. This corrinoid/iron sulfur protein transfers methyl groups from methyltetrahydrofolate to CO-dehydrogenase/acetyl-CoA synthase, a bifunctional nickel containing enzyme, which subsequently catalyzes the formation of acetyl-CoA using this methyl group, carbon monoxide, and coenzyme A (Ferry 1995)

Methyl transfer in the methane-producing Archaea

Methyl-corrinoids are essential to methane production in strictly anaerobic methane-producing bacteria (Ferry 1993). Corrinoids serve to transfer methyl groups from methanogenic substrates to coenzyme M, a methanogen-specific enzyme. Different enzymes mediate methyl transfer from methanogenic substrates such as acetate (Ferry 1992), methylamines (Burke and Krzycki 1995), methanol (Keltjens and Vogels 1994), pyruvate (Bock et al. 1994), and methyltetrahydromethanopterin, an intermediate of methane-production from formate and CO₂ (Poirot et al. 1987, Thauer et al. 1993). This last reaction is comparable to methionine synthesis in that the methyl group is transferred from an intermediate pterin to a thiol group via methyl-B₁₂ (Poirot et al. 1987)

Ribonucleotide reductases

Ribonucleotide reductase catalyzes the conversion of ribonucleotides to the deoxyribonucleotides required for DNA synthesis. Currently, four classes of reductase are known, each with their own different cofactor requirement and quaternary structure. The Ado-B₁₂-dependent reductases belong to Class II and are usually found among microorganisms (Blakley and Barker 1964, Blakley 1965). Interestingly the reaction mechanism and active site of class II reductases appear very similar to the Class I reductase of *E. coli* (Booker et al. 1994), which uses means other than Ado-B₁₂ to generate the free-radical required for catalysis (Reichard 1993).

Degradation of recalcitrant compounds

Corrinoids play an important role in the anaerobic degradation of β-hydroxy ethers and hydroxy amines such as polyethylene glycol (Frings et al. 1992), and triethanolamine (Frings et al. 1994), which are generally thought to be recalcitrant to degradation in the absence of oxygen. In addition, past studies have suggested it is possible that the

degradation of phenoxyethanol occurs in a B₁₂-dependent manner (Frings and Schink 1994), and a recent investigation suggests that a reaction similar to that seen in Ado-B₁₂-dependent diol dehydratase is involved in the process; however no corrinoid compound has been identified thus far (Speranza 2000).

Methylmalonyl-CoA mutase

Methylmalonyl-CoA mutase (MCM) catalyzes the interconversion of (R)-methylmalonyl-CoA and succinyl-CoA, and is the only carbon skeleton rearrangement that is shared in bacteria and higher animals (Flavin and Ochoa 1957, Overath et al. 1962, Allen et al. 1963, Kellermeyer et al. 1964, Fenton et al. 1982). In higher animals, this enzyme is required for the degradation of odd-chain-length fatty acids and certain branched-chain amino acids. Deficiencies in this enzyme, or those required for transport and conversion of B₁₂-precursors to Ado-B₁₂, lead to methylmalonic acidemia in humans (Ledley 1990, Allen et al. 1993, Qureshi et al. 1994). This disease is characterized by neuropsychiatric symptoms and if left untreated is often fatal during infancy. In some bacteria, MCM is involved in the fermentation of succinate to propionate, and in *Streptomyces cinnamonensis* it may be involved in the synthesis of polyketide antibiotics (Birch et al. 1993).

***S. enterica* as a Model Organism for the Study of B₁₂ Physiology and Metabolism**

To improve our understanding of B₁₂ metabolism, *Salmonella enterica* has been used as a model organism to conduct molecular genetic investigations. *S. enterica* provides an ideal system to study many aspects of B₁₂ physiology and metabolism: transport, biosynthesis, and its use as a cofactor in intramolecular rearrangements and methyl transfer can all be studied in this one organism. In addition, *S. enterica* is amenable to genetic manipulation and many methods are already in place including P22

phage transduction, expression plasmids, and an allele replacement system that uses linear transformation (Datsenko and Wanner 2000). In this dissertation studies of Ado-B₁₂-dependent PD degradation by *S. enterica* are used to improve our general understanding of B₁₂.

B₁₂-Dependent PD Degradation in *S. enterica*

Degradation of Propanediol

1,2-propanediol

Enteric bacteria encounter PD frequently, as it is a fermentation product of the methylpentoses, rhamnose and fucose, which are prevalent in the soil and in the intestinal environment (Lin 1987, Obradors et al. 1988). In addition, fucose is a component of glycoconjugates found on the surface of intestinal epithelial cells where they participate in host-parasite interactions (Bry et al. 1996). *In vivo* expression technology (IVET) has suggested that *pdu* genes are important for growth in host tissues, and competitive index studies in mice have shown that *pdu* mutations confer a virulence defect (Conner et al. 1998, Heithoff et al. 1999). The catabolism of PD in salmonella occurs in an Ado-B₁₂-dependent fashion and the genes required for this process map at the PD utilization (*pdu*) operon (Jeter 1990) ([Figure 1-3](#)).

The *pdu* locus

The *pdu* locus is situated adjacent to the *cob* operon and is comprised of the *pduF* and *pocR* genes which encode a PD diffusion facilitator and a transcriptional regulator, respectively, and the divergently transcribed *pdu* operon ([Figure 1-3](#)) (Chen et al. 1994, Bobik et al. 1997). Previously, our laboratory established the DNA sequence of the *pdu* operon and showed that it included 21 putative genes (Bobik et al. 1999). The *pduCDE* genes have been shown to encode the large, medium, and small subunits of diol

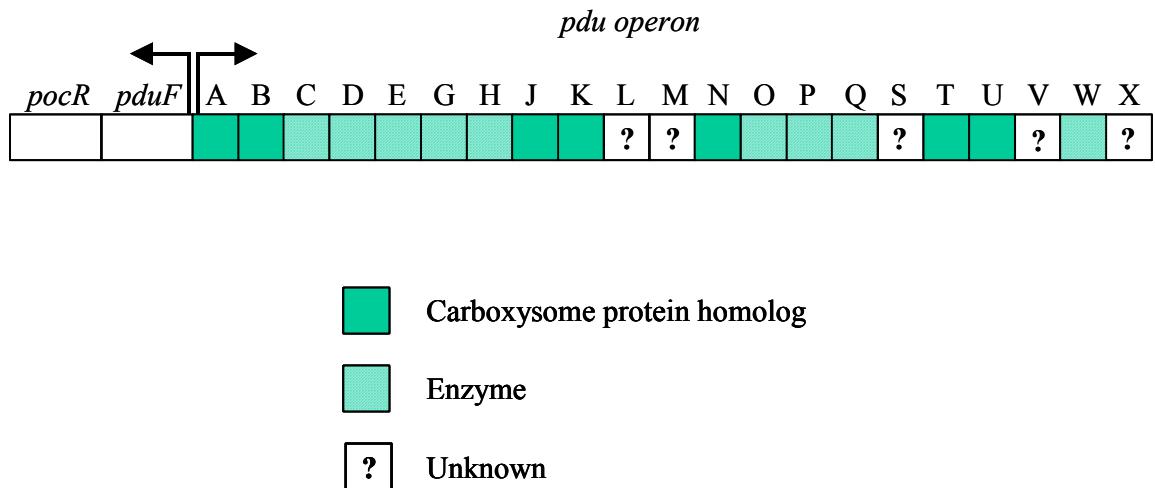


Figure 1-3. The *pdu* operon. The genes of the *pdu* operon are divided into three groups indicated by the shading of the boxes. The functions of the *pduABCDEGHJKO* and *pduP* genes have been assigned based on homology to known proteins as well as the results of biochemical and genetic studies. The functions of the remaining proteins are based on homology alone.

dehydratase, respectively; the *pduGH* genes encode proteins that are homologous to the DdrAB proteins, the two subunits of a reactivating factor for glycerol dehydratase in *Klebsiella*, the *pduO* gene has been shown to encode an adenosyltransferase, the *pduLMSVX* genes encode proteins with no convincing matches in the NCBI-nr database, and surprisingly seven genes, *pduABJKNTU*, encode products related to proteins required for the formation of the carboxysome, an organelle involved in CO₂ fixation ((Bobik et al. 1999)).

Regulation

The regulation of the *pdu* operon has been demonstrated to be quite complex (Figure 1-4). It is coregulated with the adjacent, divergently transcribed cobalamin synthetic operon (*cob*) and is controlled by both local and global regulatory systems. Locally, the positive regulatory protein, PocR, binds the P1, P2, P_{pdu}, and P_{cob} promoters when bound by its coeffectector PD, inducing expression (Chen et al. 1995). Globally both the CRP/cAMP and the two-component ArcA/ArcB system, which sense carbon availability and environmental redox potential, respectively, exert control on the *pdu/cob* regulon (Escalante and Roth 1987, Andersson 1992, Ailion et al. 1993, Chen et al. 1995). The genes required for catabolism of PD and biosynthesis of the required cofactor, Ado-B₁₂, are co-induced both aerobically and anaerobically in *S. enterica*. Aerobic induction of these genes does not require the involvement of the ArcA/ArcB system but is solely reliant upon the CRP/cAMP system. Anaerobically the ArcA/ArcB system enhances expression of the *pdu* genes, and under these conditions the ArcA/ArcB and CRP/cAMP have an additive effect on expression. Studies have demonstrated that maximal induction of the *pdu* and *cob* operons occurs under anaerobic conditions during growth on poor carbon sources in the presence of PD (Bobik et al. 1992, Chen et al. 1995).

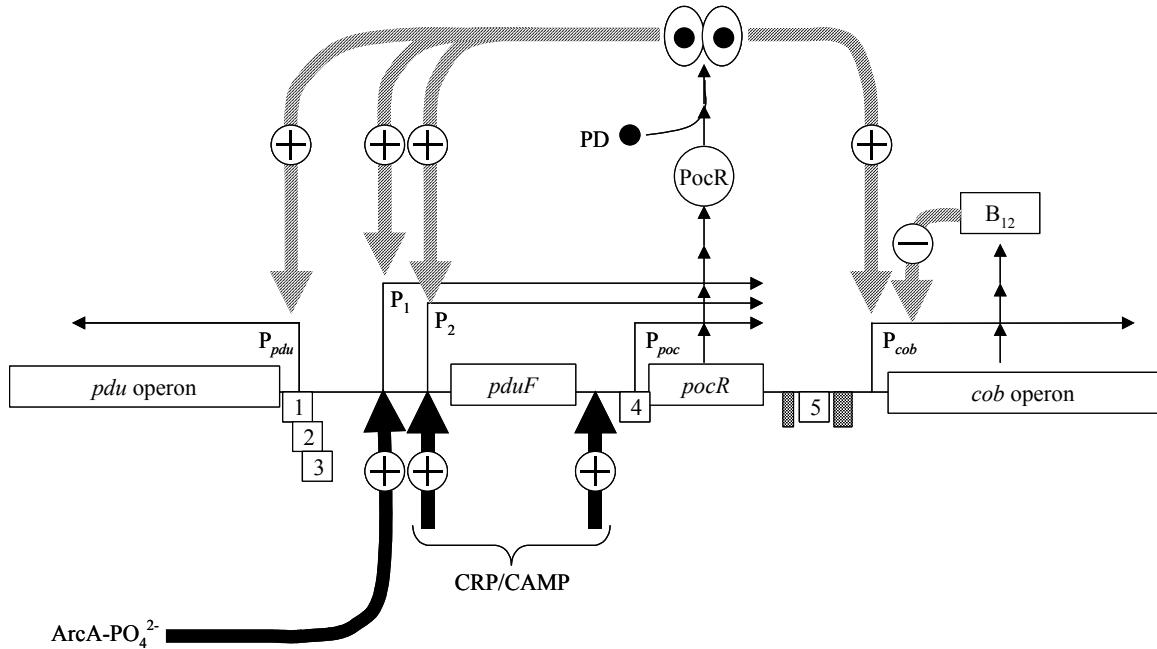


Figure 1-4. Regulation of the *pdu/cob* regulon. The hatched arrows represent local regulatory systems dependent upon either PD and or B₁₂ as indicated. The solid arrows represent regulation by global regulatory systems. The numbered boxes, 1-4, represent putative binding sites for IHF while box number five represents a known IHF binding site. The checkered boxes indicate binding spots for the PocR protein which is represented in its active form as a dimer based solely on analogy to other proteins belonging to the AraC/XylS class.

The pathway of PD degradation in *S. enterica*

The degradation of PD has been investigated (Abeles and Lee Jr. 1961, Obradors et al. 1988). The putative pathway is shown in [Figure 1-5](#). The process is initiated by the conversion of PD to propionaldehyde, mediated by the B₁₂-dependent enzyme, diol dehydratase. Currently it is thought that propionaldehyde is subsequently catabolized to propionic acid by CoA-dependent aldehyde dehydrogenase, phosphotransacetylase, and propionate kinase; and propanol by alcohol dehydrogenase (Toraya et al. 1979, Obradors et al. 1988). This pathway provides a source of ATP, an electron sink, and 3-carbon compounds which can be channeled to central metabolism via the 2-methyl-citrate pathway (Horswill and Escalante-Semerena 1997, Tsang et al. 1998). In the 2-methyl-citrate pathway, propionyl-CoA is joined to oxaloacetate, which is then subsequently converted to succinate and pyruvate. Pyruvate is then converted to acetyl- CoA and enters the TCA cycle (Witt et al. 1994, Horswill and Escalante-Semerena 1997, Textor et al. 1997, Walter et al. 1997, Tsang et al. 1998).

Under anaerobic conditions PD does not serve as a sole carbon and energy source for *S. enterica*. In theory, energy could be provided by the conversion of propionyl-phosphate to propionate and ATP and reducing power could be regenerated by the concomitant reduction of propionaldehyde to propanol which is subsequently excreted. However, in *Salmonella* fermentation of PD does not provide a carbon source, and anaerobic growth on this small molecule only occurs when dilute yeast extract is included in the growth medium (Price-Carter et al. 2001). Until recently, this phenomenon seemed paradoxical as *S. enterica* only synthesizes B₁₂ de novo under anaerobic conditions. The discovery that tetrathionate could be used as a terminal electron acceptor that would support

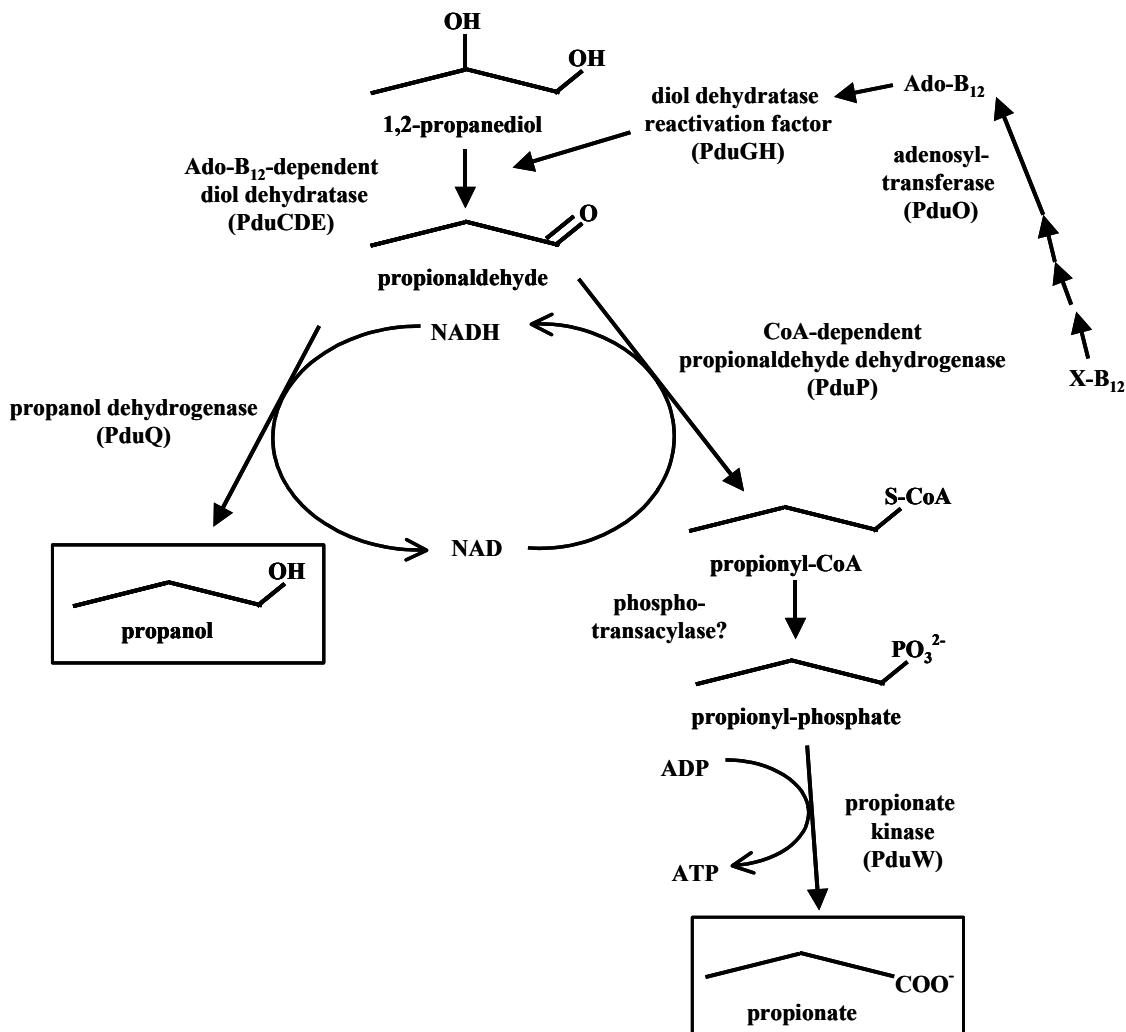


Figure 1-5. Putative pathway of aerobic PD degradation in *S. enterica*. As described in the text, conversion of propionaldehyde to propanol provides a sink for electrons generated from propionyl-CoA production and ATP is generated from the conversion of propionyl-phosphate to propionate. Propionate is thought to enter the TCA cycle via the methyl-citrate pathway (Tsang et al. 1998). The *S. enterica* proteins that encode the enzymes are listed next to each reaction.

anaerobic respiration of PD solved this paradox and provided the first conditions under which mutants unable to synthesize B₁₂ displayed a phenotype.

Homologues of Carboxysomal Proteins

Upon examination of the DNA sequence of the *pdu* operon, seven homologues of proteins required for carboxysome formation were found, *pduABJKNTU*. Both the PduA and PduJ proteins share at least 54% identity with Csos1A, while PduK and PduT share 27% amino acid identity with Csos1A (Bobik et al. 1999, Cannon et al. 2001). The *pduBNTU* genes are distantly related to genes involved in carboxysome formation (Bobik et al. 1999, Cannon et al. 2001). These findings suggested that a polyhedral organelle related to the carboxysome might be involved in Ado-B₁₂-dependent PD degradation by *S. enterica*.

The Carboxysome

Jensen and Bowen first noticed polygonal inclusion bodies in *Nostoc pruniforme* in 1961 (Jensen and Bowen 1961), and Shively et al. (1970) observed similar structures in thin sections of thiobacilli in 1970. The bodies were isolated from *Halothiobacillus neapolitanus* (formerly *Thiobacillus neapolitanus*), shown to contain ribulose bisphosphate carboxylase/oxygenase (RuBisCO), and deemed carboxysomes by Shively et al. in 1973 (Shively et al. 1973a). Since then carboxysomes have been found in all cyanobacteria thus far examined and many chemoautotrophs including several *Nitrobacter* spp., *Nitrosomonas* spp., *Prochloron* spp., *Prochlorothrix* spp., *Synechococcus* spp., *Synechocystis* spp., *H. neapolitanus* and *Thiomicrospira thyasirae* DSM5322 (formerly *Thiobacillus thyasiris*). However, carboxysomes have been isolated to some degree of purity only from *Synechococcus* PCC7942, *H. neapolitanus*, and *T. thyasiris* (Cannon and Shively 1983, Holthuijzen et al. 1986, Codd 1988).

Components of the Carboxysome

The best-studied carboxysomes are those of *Halothiobacillus neapolitanus*. They are composed of nine major proteins: the large (CbbL) and small (CbbS) subunits of RuBisCO, six shell proteins: Csos1A,B, and C, Csos2A and B, Csos3, and one protein of unknown function (Cannon and Shively 1983, Holthuijzen et al. 1986). Csos1A,B, and C are nearly identical in amino acid sequence and are encoded by a highly conserved three gene repeat which appears to be the result of a gene duplication event (English et al. 1994). Csos2 exists in two forms: Csos2A and Csos2B, which differ in their amount of posttranslational glycosylation (Baker et al. 1999). Csos3 whose presence was initially missed on Coomassie-stained gels has also been demonstrated to be a part of the carboxysome shell (Baker et al. 2000). Csos1 appears to be the most prevalent shell protein, followed by the two forms of Csos2 while Csos3 appears to be a minor shell protein. The presence of carbonic anhydrase in the carboxysomes of the cyanobacteria, *Synechococcus* PCC7942, has been reported. This finding however, has been contested by others who have attributed the presence of carbonic anhydrase activity to contamination of the preparations (Shively et al. 1998). Still others think that differences in the carboxysomes of the cyanobacteria and chemoautotrophs could account for this disparity, suggesting a scenario where carbonic anhydrase is present in the carboxysomes of cyanobacteria and absent from those of the thiobacilli ((Badger and Price 2003)). In all cases, however, no other Calvin cycle enzymes have been reported to be associated with the carboxysome.

Function of the Carboxysome

Though not entirely understood, it is thought that the carboxysome is actively involved in concentrating CO₂ and may be part of a larger CO₂-concentrating mechanism

(CCM) (Price et al. 1998, Badger and Price 2003). Mutants unable to synthesize carboxysomes required high CO₂ levels for autotrophic growth (Price and Badger 1989). In an elaborate experiment by Price and Badger (1989), it was demonstrated that expression of human carbonic anhydrase in the cytosol of the *Synechococcus* PCC7942 resulted in a massive CO₂ leak and an associated high CO₂-requiring (HCR) phenotype. It was also demonstrated that by inhibiting the carbonic anhydrase the leak was stopped. Based on these results, they composed a model of cyanobacterial carboxysomes, whereby both carbonic anhydrase and RuBisCO are sequestered in the protein shell of the carboxysome in such a manner that RuBisCO molecules surround a central core composed of carbonic anhydrase. According to their model, bicarbonate as well as CO₂ is transported into the cell, however during transport, CO₂ is converted to bicarbonate, which is the only carbon species that enters the carboxysome. Once inside the carboxysome, the formation of CO₂ from bicarbonate is catalyzed by carbonic anhydrase and CO₂ is subsequently fixed by RuBisCO as it diffuses away from the center of the carboxysome. The CO₂ leak caused by carbonic anhydrase expression in the cytosol was interpreted to be a result of bicarbonate being converted to CO₂ before it entered the carboxysome.

Research Overview

S. enterica was shown to form polyhedral organelles that resemble carboxysomes during growth on PD ((Bobik et al. 1999)). Although visually similar to the carboxysome, these polyhedral organelles are unlikely to play a role in CO₂-concentration as *S. enterica* is not an autotroph and does not express RuBisCO. In several enteric bacteria such as *Klebsiella*, *E. coli*, and *Salmonella spp.* these polyhedral organelles are associated with the B₁₂-dependent degradation of either ethanolamine, PD

or both of these compounds (Shively et al. 1998, Bobik et al. 1999, Havemann et al. 2002). Here, *S. enterica* is used as a model organism to study the role of these structures and determine what role they play in B₁₂-dependent metabolism.

The first part of this study investigated the conditions required for, and identified some of the genes involved in, the formation of the polyhedral organelles in *S. enterica*. In order to determine the conditions required for polyhedral organelle formation, several different growth substrates and conditions were tested. Formation of polyhedral organelles was found in cells grown on poor carbon source in the presence of PD both aerobically and anaerobically. Once the conditions were determined, a time course of their formation was performed during growth on PD minimal medium using electron microscopy to observe the polyhedral organelles. Once it was established when the polyhedral organelles were formed, a genetic screen was conducted using a pool of insertion mutants that localized to the *pdu* operon, and the ability to form polyhedral organelles was examined in select strains.

The second part of this study investigated the function of the PduA protein in polyhedral organelle formation. The *pduA* gene was cloned and overexpressed, and then the protein product was purified and used to generate polyclonal antiserum for use in immunolabeling studies in the wild type strain as well as in a precise, non-polar *pduA* deletion mutant. Complementation studies were also performed to further examine the role of the *pduA* gene in polyhedral organelle formation. Lastly, physiological studies were conducted to determine the effect of a *pduA* mutation on the utilization of PD and B₁₂.

As demonstrated by studies of the carboxysome, study of the polyhedral organelles of *S. enterica* would be facilitated by a purification procedure that provides homogenous, stable organelles. The final part of this study dealt with the development of such a purification procedure. Subsequent to purification the components of the organelles were separated using one and two-dimensional gel electrophoresis. SDS-PAGE, N-terminal sequencing, MALDI-TOF analysis, and immunoblotting were then used to identify the constituent proteins of the polyhedral organelles.

CHAPTER 2
ANALYSES OF POLYHEDRAL ORGANELLE FORMATION IN *Salmonella enterica*
SEROVAR TYPHIMURIUM LT2

Introduction

Virtually all salmonella degrade PD in a coenzyme B₁₂-dependent fashion, and this ability (which is absent in *E. coli*) is thought to be an important aspect of the *Salmonella*-specific lifestyle (Jeter 1990). PD is likely to be encountered by *Salmonella* in the host organism's gut, as it is a fermentation product of the plant sugars fucose and rhamnose (Lin 1987, Obradors et al. 1988). Additionally, fucose is a constituent of glycoconjugates found on intestinal epithelial cells, where it is involved in host-parasite interactions (Bry et al. 1996). *In vivo* expression technology (IVET) has indicated that the PD utilization (*pdu*) genes of *S. enterica*, may be important for growth in host tissues, and competitive index studies in mice have shown that *pdu* mutations confer a virulence defect (Conner et al. 1998, Heithoff et al. 1999). Hence, the degradation of PD appears to play an important role in the interaction of *Salmonella* with its host organisms.

The catabolism of PD has been investigated (Toraya et al. 1979, Obradors et al. 1988). The process is initiated by the conversion of PD to propionaldehyde, a reaction catalyzed by the B₁₂-dependent diol dehydratase (Abeles and Lee Jr. 1961). The aldehyde is then disproportionated to propanol by a putative propanol dehydrogenase, and to propionic acid, presumably by a CoA-dependent propionaldehyde dehydrogenase, phosphotransacylase, and propionate kinase. This pathway provides a source of energy, an electron sink and a carbon source that can be channeled into central metabolism via

the methyl-citrate pathway (Witt et al. 1994, Horswill and Escalante-Semerena 1997, Textor et al. 1997, Walter et al. 1997, Tsang et al. 1998). In *S. enterica* the fermentation of PD provides an energy source but no source of carbon while aerobic respiration and anaerobic respiration using tetrathionate as an electron acceptor allow PD to be utilized as the sole source of carbon and energy in this organism (Price-Carter et al. 2001).

In *S. enterica*, the genes required for the coenzyme B₁₂-dependent catabolism of PD cluster at the PD utilization (*pdu*) locus. This locus consists of the positive transcription regulator, *pocR*, the PD diffusion facilitator, *pduF*, and the *pdu* operon (Figure 1-3). The regulation of the *pdu* operon has been investigated previously and studies have demonstrated that the *pdu* operon is controlled by the positive transcriptional regulator, PocR and the coeffectector PD as well as the CRP/cAMP and the two component ArcA/ArcB global regulatory systems which sense carbon availability and oxygen levels in the cell environment, respectively (Bobik et al. 1992, Rondon and Escalante-Semerena 1992, Ailion et al. 1993, Rondon et al. 1995, Rondon and Escalante-Semerena 1997, Heithoff et al. 1999). The *pdu* operon has been shown to be maximally induced under anaerobic conditions during growth on poor carbon sources in the presence of PD (Bobik et al. 1999).

Recently our laboratory sequenced the *pdu* operon in its entirety and subsequent analysis suggested that it may encode a total of 21 genes; bringing the total number of *pdu* genes to 23 (Bobik et al. 1999). Six of the genes are thought to encode enzymes needed for the PD degradative pathway; two are involved in transport and regulation; two are probably involved in diol dehydratase reactivation; one is needed for the conversion

of vitamin B₁₂ (CN-B₁₂) to coenzyme B₁₂; five are of unknown function; and seven share similarity to genes involved carboxysome formation (Bobik et al. 1999).

The carboxysome is a polyhedral organelle found in cyanobacteria and some chemoautotrophs consisting of a proteinaceous shell and interior. They contain the majority of the cell's RuBisCO, a major Calvin cycle enzyme, and are thought to actively participate in concentrating CO₂. Similar bodies were observed in *S. enterica* cells grown on PD minimal medium and initial genetic analysis of the *pdu* operon indicated that genes encoded by the *pdu* operon are important for the formation of these polyhedral organelles (Bobik et al. 1999).

Here we describe investigations on the conditions needed for the formation of the polyhedral organelles. In addition, a pool of TPOP insertion mutants is screened and genetic analyses conducted on a subset of mutants indicate that both the *pduA* and *pduB* genes are required for the formation of the polyhedral organelles. Formerly, diol dehydratase was shown to be associated with the polyhedral organelles of *S. enterica* ((Bobik et al. 1999)). Here, a nonpolar diol dehydratase mutant is constructed and is demonstrated to be capable of polyhedral organelle formation. In addition, complementation studies conducted on this strain demonstrate that diol dehydratase can be packaged into the polyhedral organelles of this strain from a plasmid encoded source; however it was not determined whether diol dehydratase was packaged previous to or after the formation of the organelle shell. The results are discussed in relation to the formation and function of the polyhedral organelles.

Materials and Methods

Chemicals and Reagents

Ampicillin, fumarate, MgSO₄, pararosaniline, sodium cacodylate, sodium bisulfite,

tetrathionate, tetracycline, and vitamin B₁₂ were from Sigma Chemical Co.; St. Louis, MO. Isopropyl-β-D-thiogalactopyranoside (IPTG) was from Diagnostic Chemical Ltd.; Charlottetown, Canada. Glucose, formaldehyde (r,s), mono and dibasic potassium phosphate, ammonium sodium phosphate tetrahydrate, pyruvate, and succinate, were from Fisher Scientific; Pittsburgh, PA. Glutaraldehyde was from Tousimis; Rockville, MD. Uranyl acetate was from E.M. Sciences; Ft. Washington, PA. Osmium tetroxide was from Tedpella Inc.; Redding, CA. Powdered milk was from Nestle; Glendale, CA. Yeast extract, MacConkey agar base, agar, Luria-Bertani (LB) agar and LB broth were from Difco Laboratories; Detroit, Michigan.

Bacterial Strains, Media, and Growth Conditions

The bacterial strains used in this study are listed in [Table 2-1](#). The complex medium used was LB broth. The minimal medium used was NCE, prepared according to Vogel and Bonner (Vogel and Bonner 1956) and supplemented with 1 mM MgSO₄. Agar was added to a final concentration of 1.5% to make solid media. Carbon sources were used at the following concentrations: fumarate, 0.32%; glucose, 0.2% (aerobically) and 0.4% (anaerobically); glycerol, 0.4%; PD, 0.2 or 0.4%; pyruvate, 0.44%; and succinate, 1.0%. Tetrathionate was used at 10 mM, CNB₁₂ was used at 200 ng/ml, tetracycline at 10 µg/ml, and ampicillin at 100 µg/ml. Overnight cultures were grown aerobically at 37°C, with shaking at 275 RPM in LB broth supplemented with the appropriate antibiotics. MacConkey/PD/B₁₂ indicator plates were composed of MacConkey agar base supplemented with 1% PD and 200 ng B₁₂ per ml. Aldehyde indicator plates were prepared according to the method of Conway et al. (Conway et al.

1987) with the following modifications: pararosaniline was added to sterile medium as a fine powder, 200 ng/ml of CN-B₁₂ was included, and ethanol was replaced by 1% PD.

TPOP Mutagenesis

The Tn10dTet elements used to create nonpolar insertion mutants in the strains used in this study are transposition defective derivatives of Tn10 from which the transposase gene and the internal ends of IS10 have been deleted (Way et al. 1984). In addition, these transposons, referred to as TPOPs, have been further modified in order to increase transcription from the tetracycline inducible promoters (Rappleye and Roth 1997). Hence TPOPS are nonpolar in the presence of tetracycline but polar in its absence. For TPOP mutagenesis of the *pdu* region, a pool of approximately 80,000 independent insertion mutations was prepared as described in Bobik et al. (Bobik et al. 1992). The pool was then used as a donor in transductional crosses with strain BE235 (*pdu12::MudJ*). Tetracycline resistance was selected, and transductants were screened for loss of the *pdu12::MudJ* elements using MacConkey/lactose/PD indicator plates (Bobik et al. 1992). Following transposon mutagenesis, 30,000 colonies were screened, and 120 TPOP1 insertions located near the *pdu* operon were isolated. This work was performed by Lydia Jorge and Dr. Thomas Bobik.

Localization of TPOP Insertions

PCR was used to amplify the region of DNA that included one join-point between the TPOP element and the *S. enterica* chromosome. The primers used for amplification in strain BE33 were: forward, 5'ACCTTGTCACCAACGCTTTCC-3' and reverse, 5'-GTTCATATGCGAAACCACTTC-3'. The DNA sequence of the PCR product was determined, and the location of the downstream join-point was determined to be bp 160 of the *pduA* coding sequence. The localization of the other TPOP insertions was

determined using similar methods by Lydia Jorge, Elizabeth Mihalcik, and Dr. Thomas Bobik.

Table 2-1. Bacterial strains

Species	Strain	Genotype
<i>E. coli</i>		
	DH5α	F ⁻ λ ⁻ endA1 hsdR17 relA1 supE44 thi-1 recA1 gyrA96 Δ(lacIZYA-argF)U169 (φ80dlacZΔM15)
	HB101	F ⁻ Δ(gpt-proA)62 leuB6 glnV44 ara-14 galK2 lacYI Δ(mcrC-mrr) rpsL20 xyl-5 mtl-1 recA13
	S17.1 λpir	recA (RP4-2-Tc::Mu) λpir
	GH1	HB101/pGH1 (contains ΔpduCDE insert, Amp ^R)
	GH13	S17.1 λpir/pCVD442 (contains ΔpduCDE insert from GH1, Amp ^R)
	RT1679	DH5α/pVJ70 pduF'ABCDEG (Amp ^R) Wild type
<i>S. enterica</i> serovar Typhimurium LT2	TR6579	metA22 metE551 trpD ilv-452 hsdLT6 hsdSA29 HsdB ⁻ strA120 GalE ⁻ Leu ⁻ Pro ⁻
	BE33	pduA::T-POP11 ^a
	BE34	pduC::T-POP21 ^a
	BE35	pduC::T-POP23 ^a
	BE37	pduA::T-POP43 ^a
	BE38	pduB::T-POP78 ^a
	BE47	thr-480::Tn10dCam
	BE79	ΔpduCDE thr-480::Tn10dCam (precise nonpolar deletion of pduCDE) ^a
	BE87	ΔpduCDE
	BE113	MetE205 ara-9 Δ299/plac22 (Amp ^R)
	BE235	(pdu12::MudJ)
	GH4	pduA::TPOP11/pTA749 (pduA gene under plac control, Amp ^R)
	GH9	TR6579/pGH1 (pduB under plac control, Amp ^R)
	GH110	DELpduCDE/pXY39 (pduCDE under plac control, Amp ^R)

^aSee Materials and Methods.

Genetic Techniques

Transductional crosses were accomplished using the high-frequency generalized transducing phage P22HT105/1 int-201 (Schmieger 1971). Transductants were single colony purified on non-selective green indicator plates (Chan et al. 1972). Cross-

streaking to check phage sensitivity was done using a P22 clear-plaque mutant, H5. Electroporation of low ionic strength cell suspensions was done with a Gene Pulser (Bio-Rad; Hercules, CA) as specified by the manufacturer and used at the following settings: capacitance, 25 μ F; capacitance extender, 250 μ F; pulse controller 200 Ω ; voltage, 2.5kV using cuvettes with a 0.2 cm gap. Putative transformants were purified by single colony isolation. Plasmid DNA was isolated with Qiagen Spin mini-prep kits (Qiagen Inc., Santa Clarita, CA).

Cloning of the *pduB* Gene for Complementation Studies

Vector *plac22* was used for cloning the *pduB* gene so that its expression could be induced by IPTG (Bobik et al. 1997). The DNA used for cloning was obtained via PCR amplification of plasmid, pVJ70, using the following primers: forward 5'-
GGAATTCAGATCTATGGCAGAAAAAAGCTGCAGTTAACG-3'; and reverse, 5'-
AAGGATCCAAGCTTGAATCAGCCTCGTGGGTATCAGATG-3'. The *Bg*II (forward primer) and *Hind*III (reverse primer) sites are underlined. *Pfu* polymerase was employed for the amplification because of its high fidelity of replication. The amplified DNA was gel purified and ligated to *plac22* that had been digested with the same restriction enzymes and gel purified. The ligation was heated to 70°C for 15 minutes to inactivate the ligase and then used to transform *S. enterica* TR6579 by electroporation. A clone (pGH9) containing an insert of expected size (722 bp) was moved into BE38 by transduction to create strain GH42.

Complementation Studies

Strains were grown in batch culture at 37°C, with shaking at 275 RPM, in 125 or 250 ml Erlenmeyer flasks. NCE minimal medium supplemented with either 1%

succinate and 0.4% PD for studies performed on strains GH4, BE87, and GH110 or 0.4% PD only for strain GH42 were used as the growth media. For strains containing TPOP insertions, tetracycline was included in the medium at a concentration of 2 µg/ml to induce expression out of the TPOP insertion (Rappleye and Roth 1997). IPTG was added at various concentrations at specific times during growth as described in the text. Cells were grown to late log phase (OD₆₀₀ between 0.8 and 1.0) and processed for observation of ultrastructure or immunogold localization as described in “Electron Microscopy” and in the text.

Electron Microscopy

To examine ultrastructure, cells were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH7.2) for 30 min. at room temperature and then in 1% (w/v) osmium tetroxide in the same buffer for 1 hr at 4°C. The samples were then dehydrated through a graded ethanol series (25%, 50%, 75%, and 100% v/v), followed by replacement with absolute acetone, embedding in Spurr’s low-viscosity resin, and polymerized in microfuge tubes for 1 day at 60°C. In order to impart extra contrast to the polyhedral organelles, uranyl staining *en bloc* was performed by including 1% uranyl acetate in the 75% ethanol during the dehydration. Samples were sectioned using an LKB Nova ultramicrotome, collected on Formvar-coated 100 mesh copper grids, post-stained with uranyl acetate followed by staining with lead citrate, and then viewed and photographed on a Zeiss EM-10CA transmission electron microscope.

For immunogold localization of diol dehydratase, cells were fixed in 0.5% glutaraldehyde/4% paraformaldehyde on ice for 20 min (Aldrich et al. 1992). They were then dehydrated in a graded series of ethanol (25%, 50%, 75%, 100%), embedded in LR

white resin, and polymerized in gelatin capsules at 50°C for 5 days (Aldrich et al. 1992). Thin sections were placed on 100 mesh Formvar-coated nickel grids, blocked for 30 min. on 1% powdered milk in phosphate-buffered saline (PBS) at pH 7.2, and floated overnight on rabbit polyclonal antibody against purified diol dehydratase from *Klebsiella oxytoca* (provided by Tetsuo Toraya) diluted 1:1000 in PBS. After labeling with the primary antibody, the grids were washed two times with high-salt Tris-Tween buffer, followed by two washes with water and then floated on goat anti-rabbit antibody conjugated to 12-nm diameter colloidal gold particles (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.). Samples were washed as done for primary labeling, fixed for 10 minutes by floating on Trump's reagent (McDowell and Trump 1976), and then post-stained with 0.5% uranyl acetate followed by staining with lead citrate.

Construction of In-Frame *pduCDE* Deletion

Restriction digestion was used to delete *pduD* and fuse the remaining 5' *pduC* and 3' *pduE* DNA sequence ([Figure 2-1](#)). Plasmid pVJ70 containing the *pduABCDEFG* genes was digested with *Nru*I releasing two fragments, of 1123 and 2448 bp, between *pduC* and *pduE*, leaving 90 bp of the 5' region containing the translational start site of *pduC* and 225 bp of the 3' region containing the translational stop site of *pduE* intact. Next a 10 bp linker (GAAGATCTTC) was ligated into the digested vector in such a manner that the reading frame between the remaining *pduC* and *pduE* sequences was retained. The ligation reaction was used to transform *E. coli* HB101 via electroporation, and DNA sequencing was performed on subsequent clones to verify that only one linker had been introduced into the plasmid. One clone (plasmid pGH1) containing an insert of desired sequence, was digested with *Xba*I and *Sph*I and then ligated into pCVD442 using

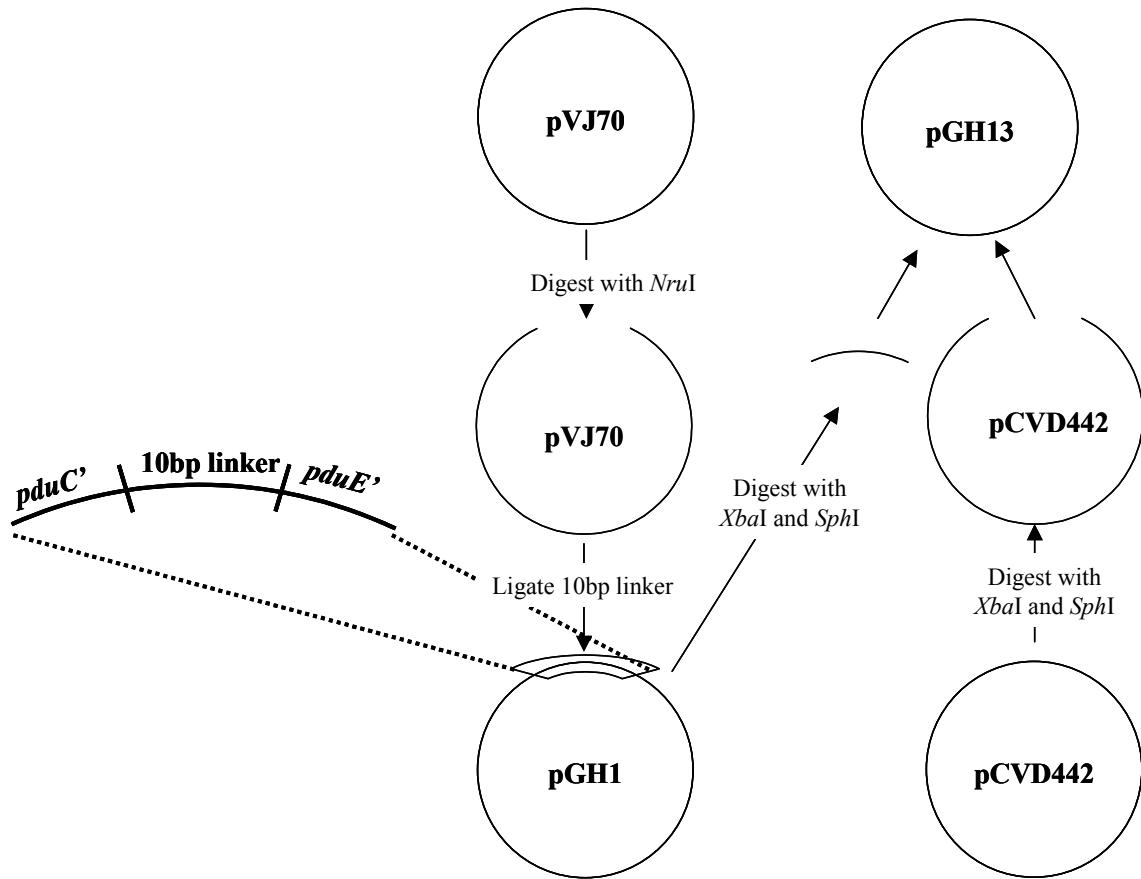


Figure 2-1. Construction of plasmid pGH13 used in generating the *pduCDE* deletion in strain BE87. Plasmid pVJ70 containing the *pduABCDEFG* genes was digested with *Nru*I. This digestion removed the entire *pduD* gene and most of the *pduC* and *pduE* genes. A 10bp oligonucleotide (linker) was then ligated between the remainder of the *pduC* and *pduE* genes to retain the reading frame and create a short fusion peptide. The resulting construct was then subcloned into the suicide vector, pCVD442, which was later used to move the *pduCDE* deletion into the *S. enterica* genome.

the same restriction sites. The ligation reaction was used to transform *E. coli* S17.1 λ pir via electroporation, and one clone (plasmid pGH13) containing an insert of expected size (3,666 bp) was used to introduce the deletion into the *S. enterica* chromosome using the procedure of Miller and Mekalanos with some modification (Miller and Mekalanos 1988). For the conjugation step, BE47 was used as the recipient, and Amp^r and Cam^r colonies were selected. Instead of the sucrose sensitivity screen used in the protocol, MacConkey/PD/CN-B₁₂ plates were used to screen for colonies that had lost the ability to degrade PD. Deletion strains were identified by PCR using whole cells as a source of template. A transducing phage lysate of LT2 was used to cross off the Cam marker in strain BE79 to make strain BE87.

Results

Polyhedral Organelles are Formed in Cells Grown on Poor Carbon Sources in the Presence of PD

In order to define the conditions necessary for polyhedral organelle formation in *S. enterica*, wild type cells were grown to late log phase in liquid media using various carbon sources under different environmental conditions and then examined for the presence of polyhedral organelles using electron microscopy. The results of the tests are displayed in [Table 2-2](#). When grown on PD, *S. enterica* formed polyhedral organelles ([Figure 2-2A](#)). This was observed during both aerobic growth on PD and during anaerobic growth on PD when tetrathionate was supplied as the terminal electron acceptor. Tetrathionate was used as an electron acceptor for respiration of PD since the more common electron acceptors nitrate, fumarate, trimethaline-N-oxide (TMAO) or dimethyl sulfoxide (DMSO) do not support growth on PD (Price-Carter et al. 2001). The only other growth conditions under which polyhedral organelle formation was observed

Table 2-2. Conditions under which polyhedral organelles are formed

Growth conditions	Aerobic or anaerobic	Polyhedra formation
Succinate/PD	Aerobic	Yes
Succinate/PD/B ₁₂	Aerobic	Yes
PD/B ₁₂	Aerobic	Yes
LB	Aerobic	No
LB/PD	Aerobic	No
Glucose ^a	Aerobic	No
Glycerol	Aerobic	No
Succinate	Aerobic	No
Pyruvate/fumarate/PD ^b	Anaerobic	Yes
PD/tetrathionate ^b	Anaerobic	Yes
Glycerol/fumarate	Anaerobic	Rare
Glycerol/tetrathionate	Anaerobic	Rare
Glucose ^a	Anaerobic	No
Pyruvate/tetrathionate ^b	Anaerobic	No
Pyruvate/fumarate/tetrathionate ^b	Anaerobic	No
Pyruvate/fumarate ^b	Anaerobic	No

^aResults provided by Dr. Thomas Bobik.

^bResults provided by Robert Busch.

was during aerobic growth on a mixture of succinate and PD or during anaerobic growth on a mixture of pyruvate, fumarate, and PD. Anaerobic growth on succinate/PD minimal medium was not performed since *S. enterica* is unable to metabolize succinate anaerobically.

To determine if catabolism of PD is a prerequisite for polyhedral organelle formation, aerobic growth of *S. enterica* on succinate/PD minimal medium was conducted in both the presence and absence of CN-B₁₂. Under aerobic conditions without CN-B₁₂ supplementation, *S. enterica* is unable to catabolize PD since de novo synthesis of B₁₂ occurs only under anaerobic conditions (Müller et al. 1990, Müller et al. 1991). Polyhedral organelles were observed in cells under both conditions indicating that the catabolism of PD is not required for the formation of the polyhedra organelles in *S. enterica*. Polyhedral organelles were not formed on LB medium supplemented with

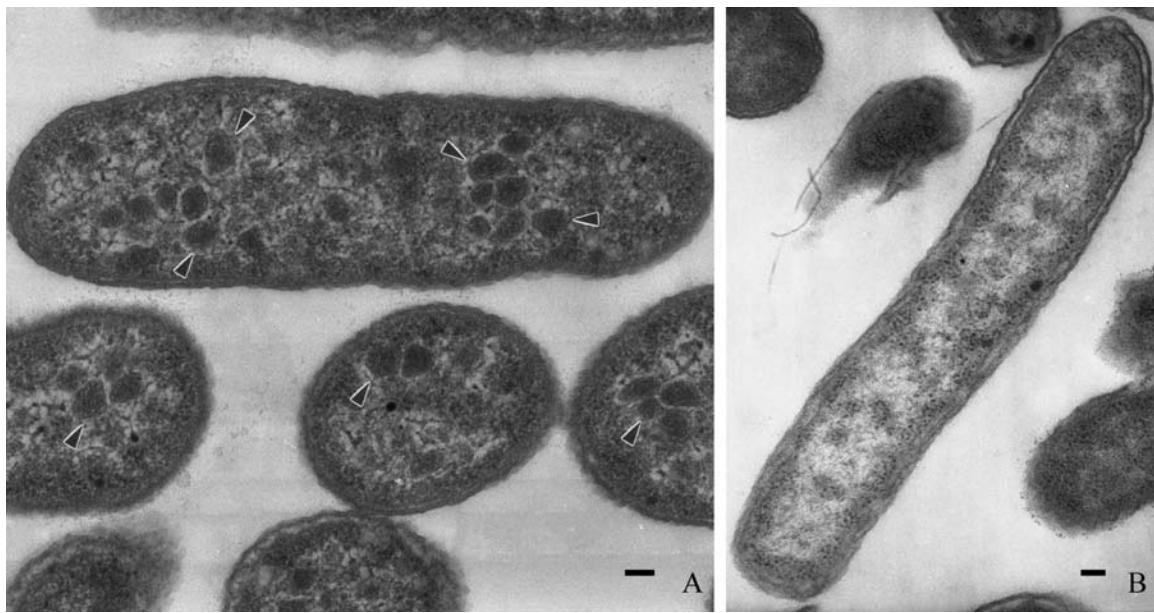


Figure 2-2. Electron micrographs of *S. enterica* grown under different conditions. A. *S. enterica* grown aerobically on PD minimal medium. B. *S. enterica* grown aerobically on glucose minimal medium. Both pictures were kindly provided by Dr. Henry Aldrich. Polyhedral organelles are present in the cells grown on PD (arrows) but not in those grown on glucose. Bars in lower right-hand corners are 100 nm in length.

level) resulted in no observable difference in polyhedral organelle formation indicating that anaerobic respiration using tetrathionate as an electron acceptor is not required for polyhedral organelle formation. Interestingly, only rarely were polyhedral organelles observed in cells grown anaerobically on glycerol using either tetrathionate or fumarate as an electron acceptor. These conditions have been demonstrated previously to highly induce the *pdu* operon and the reduction of organelle formation suggests that PD may play some role in the formation of the polyhedral organelles in addition to its role in induction of the *pdu* operon.

Time Course of Organelle Formation

The time course of polyhedral organelle formation was followed during aerobic growth of *S. enterica* on PD minimal medium ([Figure 2-3](#)). Samples were taken during different phases of growth and observed using electron microscopy. The polyhedral organelles proved difficult to discern from other cytoplasmic constituents even with the utilization of improved staining techniques designed to impart extra contrast to the polyhedral organelles (Bobik et al. 1999). To facilitate the identification of polyhedral organelles, cells were labeled with a polyclonal antiserum generated against diol dehydratase, an enzyme formerly shown to be associated with the polyhedral organelles (Bobik et al. 1999). As expected, the polyhedral organelles were not observed in the LB inoculum. After transfer to PD minimal medium, polyhedral organelles were observed in cells as early as 5 hr subsequent to the transfer. The number of polyhedral organelles was highest at this point, remained constant throughout the log phase and seemed to decrease in the remainder of the growth curve. Nevertheless, polyhedral organelles were observed in cells throughout the remainder of the growth curve and were present even in dead

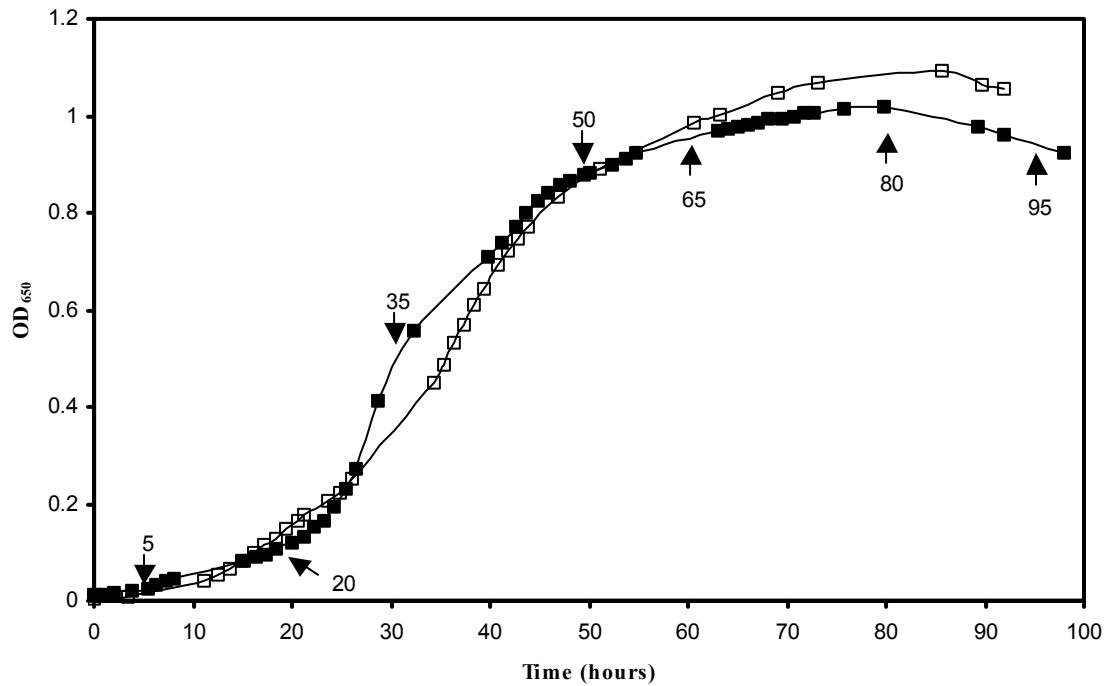


Figure 2-3. Aerobic growth of *S. enterica* on PD minimal medium. Closed squares represent growth after transfer from LB medium. Open squares represent growth after transfer to fresh PD medium from a mid log PD minimal medium culture. Arrows indicate sampling times (in hours) for electron microscopy observation.

cells. like the carboxysome no discernable stages of polyhedra assembly were noted during the time course (Orus et al. 1995).

Screening of TPOP Mutants

A pool of TPOP insertions was tested for their ability to produce acid and aldehydes and the ability to use PD as a sole carbon and energy source using MacConkey/B₁₂/PD, AIM, and PD minimal media plates, respectively. TPOP insertions are polar in the absence of tetracycline, however, when tetracycline is present expression out of both ends of the insertion is induced effectively making the insertion nonpolar (Way et al. 1984, Rappleye and Roth 1997). Five mutants were selected for further study to determine the effects of the TPOP insertions on polyhedral organelle production. The results of the study are represented in [Table 2-3](#). As expected, strains BE34 and BE35, which both carry TPOP insertions in the *pduC* gene, encoding the large subunit of diol dehydratase, were unable to use PD for growth; however, it was surprising that strains BE33 and BE37, which carry TPOP insertions in the *pduA* gene, and strain BE38, which carries an insertion in the *pduB* gene, were either unable to or exhibited a reduced ability to use PD for growth. Both the *pduA* and *pduB* genes encode proteins homologous to those required for carboxysome formation, and these strains were of particular interest since their inability to synthesize the polyhedral organelles suggests an effect on growth using PD.

Effect of TPOP Insertions on the Formation of Polyhedral Organelles

The ability of the five insertion mutants to form polyhedral organelles was examined ([Table 2-3](#)). Strains BE33-BE37 containing TPOP insertion mutations were grown on succinate/PD minimal medium containing tetracycline to induce expression of genes downstream of the TPOP insertion. Cells were grown to late log phase, harvested,

Table 2-3. Acid production, aldehyde production, growth on minimal PD media, and polyhedra production in a subset of TPOP insertion mutation strains.

Strain	Relevant Genotype	Approx. location of TPOP ^a	Growth on PD	Aldehyde production	Acid production	Polyhedra production
LT2	wild type	N/A	+	+	+	+
BE33	<i>pduA</i>	bp 2605 <i>pdu</i> operon	-	+/-	+/-	-
BE37	<i>pduA</i>	bp 2367 <i>pdu</i> operon	-	+	+/-	-
BE38	<i>pduB</i>	bp 2978 <i>pdu</i> operon	+/-	+/-	+	-
BE34	<i>pduC</i>	bp 5021 <i>pdu</i> operon	-	-	+/-	+
BE35	<i>pduC</i>	bp 4616 <i>pdu</i> operon	-	-	+/-	-

^a0 bp is the start of the *pocR* gene. N/A, not applicable.

and observed using electron microscopy. No polyhedral organelles were observed in cells of strains BE33 and BE37, which contained TPOP insertions in the *pduA* gene. Likewise, no polyhedra were observed in strain BE38 containing a TPOP insertion in the *pduB* gene. When strains BE34- BE36 containing TPOP insertions in the *pduC* gene were examined, no polyhedra were observed in cells of strain BE35 or BE36; however, cells of strain BE34 appeared to still be capable of forming polyhedral organelles. The TPOP insertion in this strain mapped further downstream in the *pduC* gene than that in strain BE35 suggesting that the C-terminus of the PduC protein is not required for polyhedral organelle formation.

Complementation of the Polyhedra Organelle-Negative Phenotype of *pduA* and *pduB* TPOP Insertion Mutants

Strain GH4 was constructed by moving plasmid, pTA749 (*plac22-pduA*), into strain BE33 containing a TPOP1 insertion in the *pduA* gene. To determine whether the *pduA* gene present on the plasmid could complement the *pduA*::TPOP11 for polyhedral

body formation, strain GH4 was grown on succinate/PD minimal medium using several IPTG concentrations and then examined by electron microscopy. Strain GH4 did not synthesize polyhedral organelles at 0.1 mM IPTG ([Figure 2-4B](#)) and instead rod-like structures were observed which became more prevalent at 1 mM IPTG ([Figure 2-4C](#)). In the absence of IPTG, no normally shaped organelles or rod-like structures were formed ([Figure 2-4A](#)). A similar phenomenon was noted when complementation of strain GH42 containing a TPOP1 insertion in the *pduB* gene and carrying plasmid pGH2, encoding the *pduB* gene was attempted. Aberrant structures were produced at 0.1 mM IPTG ([Figure 2-5B](#)) which became more prevalent at 1 mM IPTG ([Figure 2-5B](#)) and no organelles or aberrant structures were observed in the absence of IPTG ([Figure 2-5C](#)). The finding that GH4 and GH42 produced aberrant structures in the presence of IPTG but not in its absence suggests that PduA and PduB may play a role in organelle formation. It also suggests that downstream expression from the tetracycline-inducible promoters in the TPOPs is not similar enough to wild type levels to produce a normal appearing organelle.

The B₁₂-Dependent Diol Dehydratase is not Required for the Formation of the Polyhedral Organelles

Strain BE87, containing a nonpolar deletion of the *pduCDE* genes, was found to be unable to degrade PD using MacConkey and AIM plates and no growth was observed on PD minimal medium plates. The strain was grown on PD/succinate minimal medium and observed using electron microscopy. Polyhedral organelles were observed in this strain ([Figure 2-6](#)) and when similarly prepared samples were labeled with polyclonal antibody against diol dehydratase, no diol dehydratase was found to be associated the organelle or the cell ([Figure 2-7C](#)). The interiors of the polyhedral organelles of this strain appear

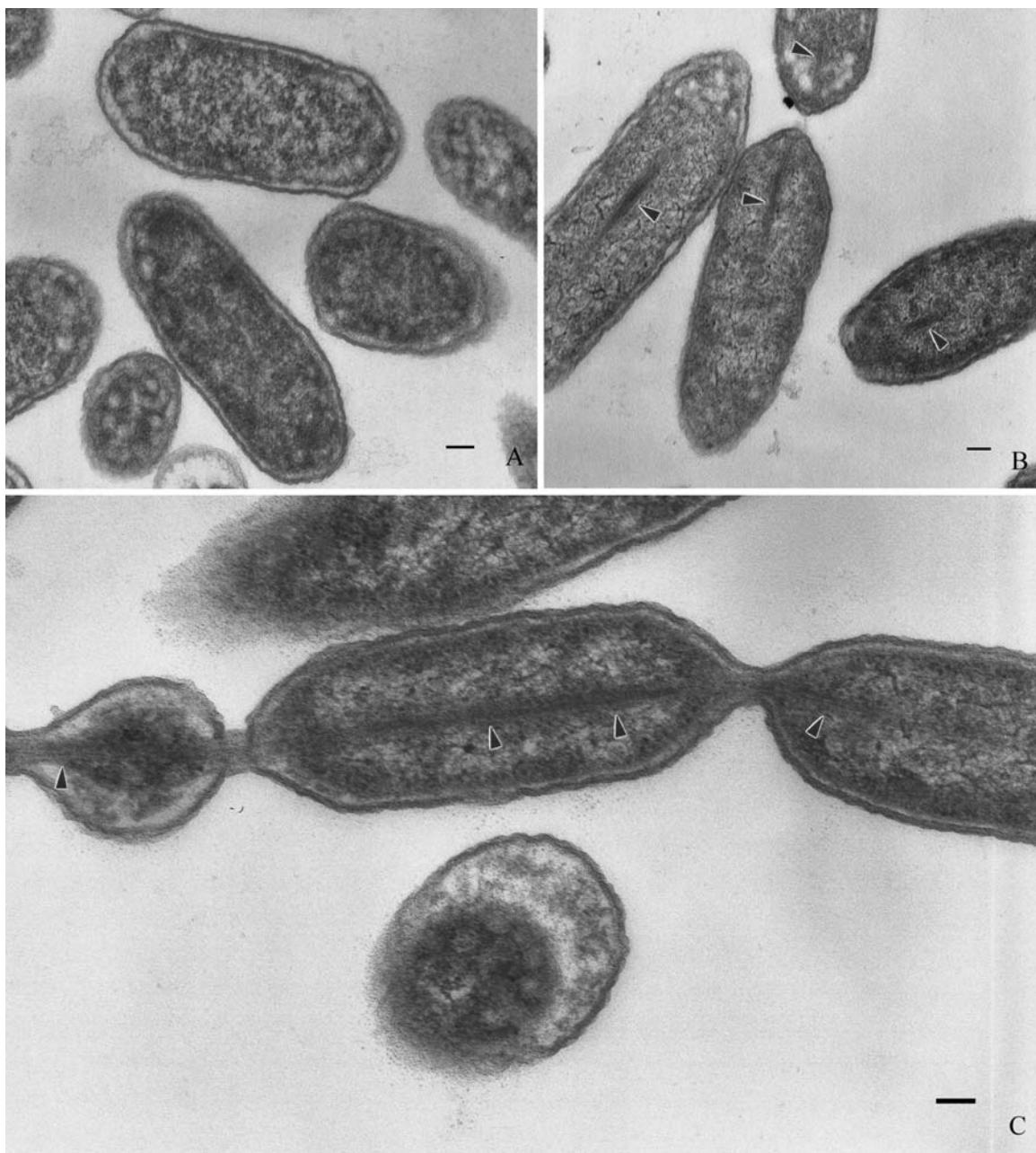


Figure 2-4. Complementation of a *pduA*::TPOP insertion mutant. Cells were grown on succinate/PD minimal media containing tetracycline to induce expression downstream of the TPOP insertion. IPTG was included at the following concentrations to induce expression of *pduA* from a plasmid: Panel A, 0 mM; Panel B, 0.1 mM, Panel C, 1.0 mM. Arrowheads indicate the position of the rod-like structures. Bars in lower right-hand corners are 100 nm in length.

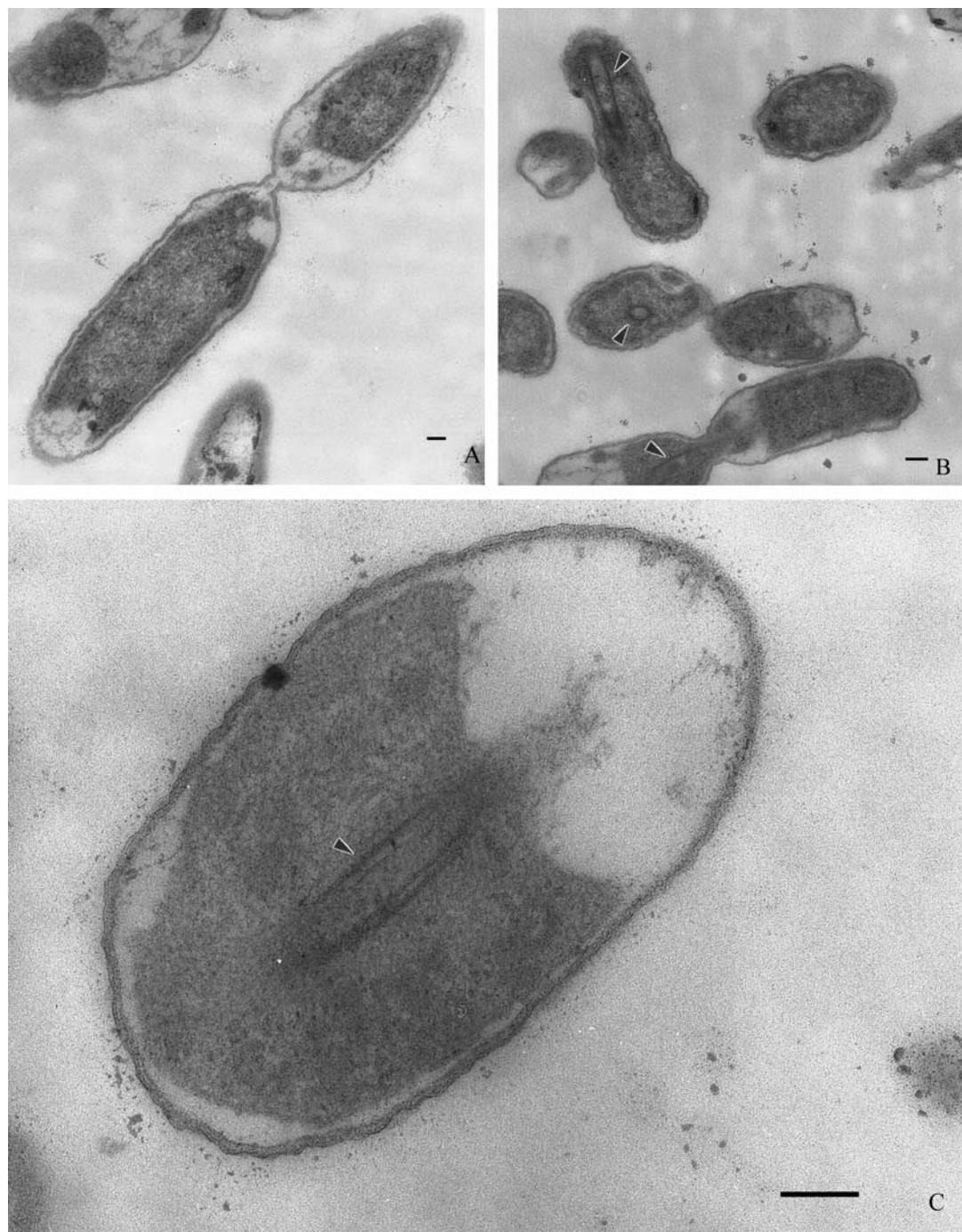


Figure 2-5. Complementation of a *pduB*::TPOP insertion mutant. Cells were grown on PD minimal media containing tetracycline to induce expression downstream of the TPOP insertion. IPTG was included at the following concentrations to induce expression of *pduB* from a plasmid: Panel A, 0 mM; Panel B, 0.1 mM; Panel C, 1.0 mM. Arrowheads indicate the position of the aberrant structures. Bars in lower right-hand corners are 100 nm in length.

different than those found in the wild type (compare [Figure 2-6](#) and [Figure 2-2A](#)) but still appear to have some proteinaceous contents.

Localization of Plasmid Encoded Diol Dehydratase in a *ΔpduCDE* Mutant

To examine the order of diol dehydratase insertion into the organelle, strain GH110 was constructed by moving plasmid, pXY39, containing the *pduCDE* genes under control of an IPTG inducible promoter, into strain BE87 (*ΔpduCDE*) via transduction. The localization of diol dehydratase was then examined in cells where expression of diol dehydratase was induced immediately after transfer from rich to PD/succinate minimal medium (before polyhedral organelle formation) ([Figure 2-7A](#)) and in cells where expression of diol dehydratase was induced in a mid-log succinate/PD minimal media culture (after polyhedral organelle formation) ([Figure 2-7B](#)). In both of these cases some diol dehydratase localized to the polyhedral organelles and no labeling was observed in a control strain without plasmid ([Figure 2-7C](#)). Also in both instances large polar bodies appearing to be entirely composed of diol dehydratase were observed. The presence of these polar bodies is most likely due to the excessive amounts of diol dehydratase that were expressed. Based on these results it is difficult to determine if diol dehydratase is packaged into the polyhedra before or after formation of the polyhedral shell since diol dehydratase is not required for organelle formation (see above) and nothing is known about the turnover of these organelles, thus leaving the possibility that diol dehydratase is being incorporated into organelles formed during mid-log phase.

Genes Downstream of Diol Dehydratase are Required for the Formation of Polyhedral Organelles

Aberrant polyhedral organelles were observed in strain RT818 containing a polar insertion downstream of the *pduG* gene ([Figure 2-8](#)) (Bobik et al. 1999). This suggests

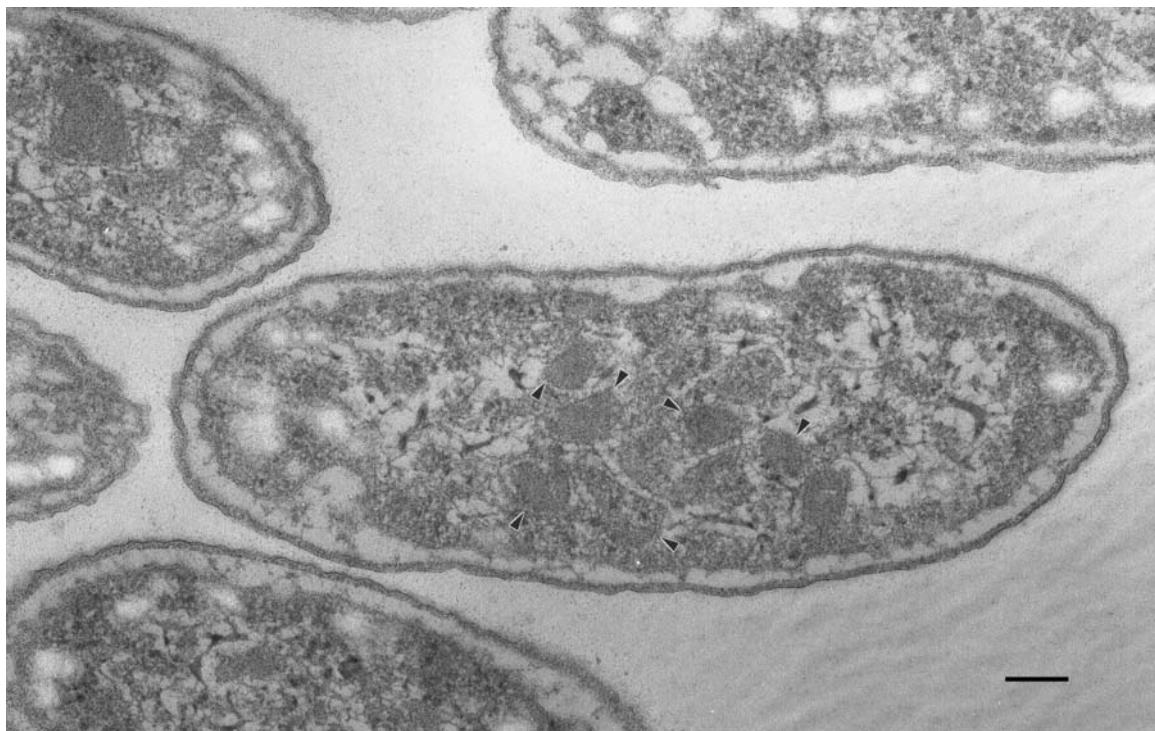
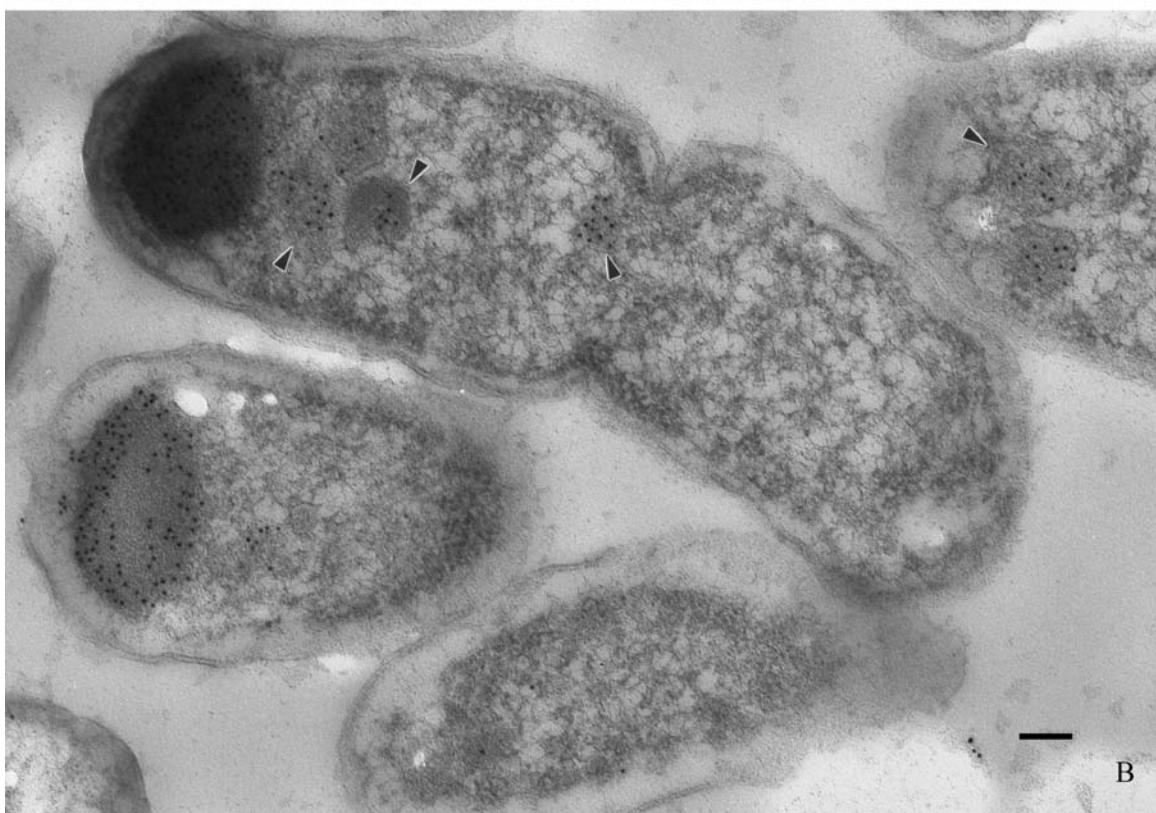
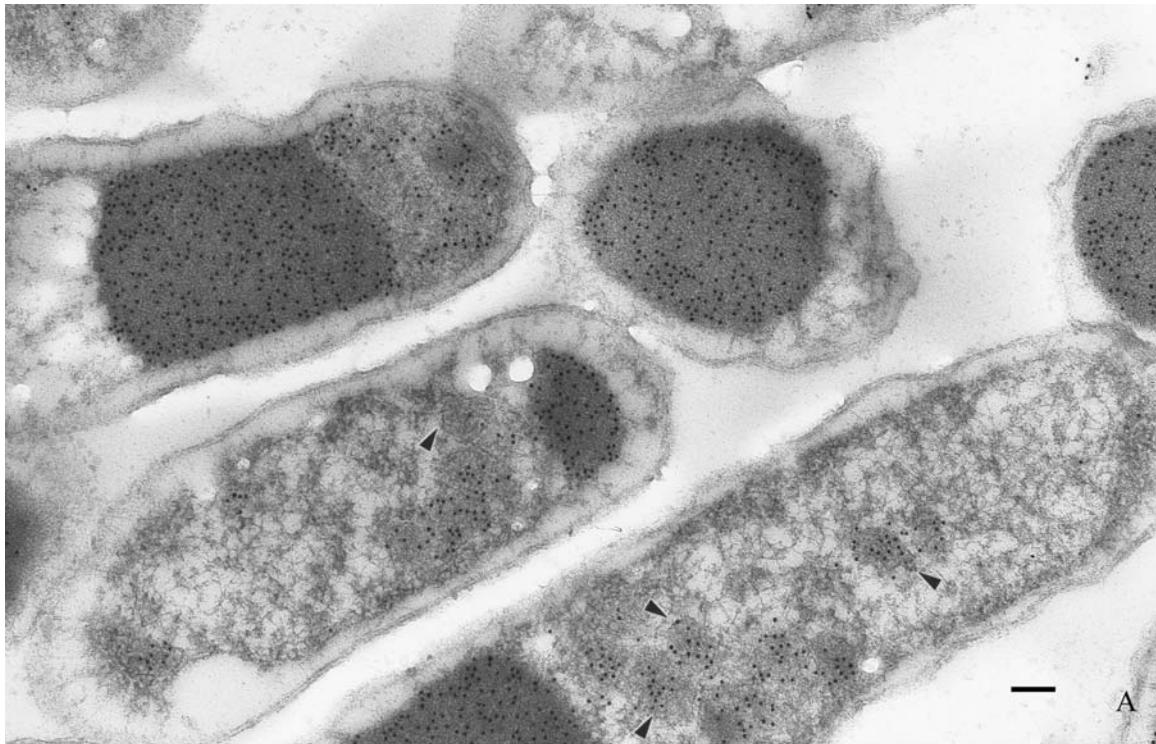


Figure 2-6. Electron micrograph of a $\Delta pduCDE$ mutant grown on succinate minimal medium in the presence of PD. Note the presence of polyhedra and the difference of the staining as compared to that in the wild type strain ([Figure 2-2](#)). Arrows indicate the position of the polyhedral organelles. The bar in the lower right-hand corner is 100 nm in length.



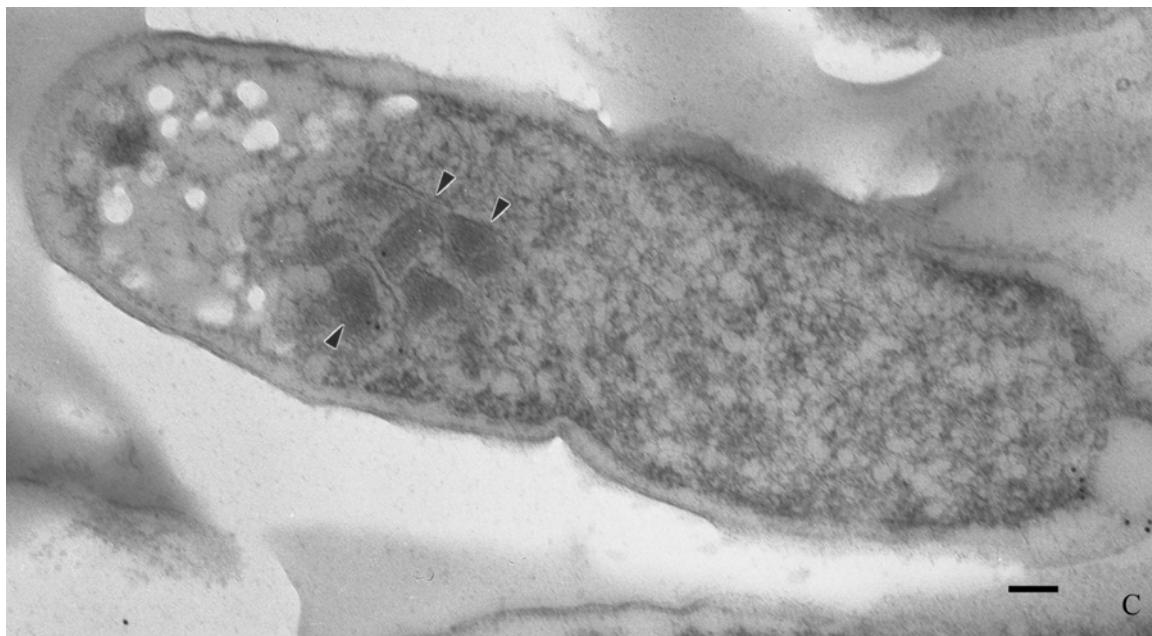


Figure 2-7. Packaging of diol dehydratase. The location of diol dehydratase was examined in strain GH110, a diol dehydratase mutant that carries plasmid pXY39 encoding the three subunits for diol dehydratase using immunolabeling with anti-diol dehydratase antiserum. The location of diol dehydratase was examined in strains where diol dehydratase expression had been induced both prior to and subsequent to polyhedral organelle formation. Panel A, diol dehydratase expression induced before formation of the polyhedral organelles; Panel B, diol dehydratase expression induced subsequent to organelle formation. Panel C, diol dehydratase mutant (BE87). Gold particles (black dots) indicate the position of diol dehydratase and arrowheads indicate the position of the polyhedral organelles. The bar in the lower right-hand corner is 100 nm in length.

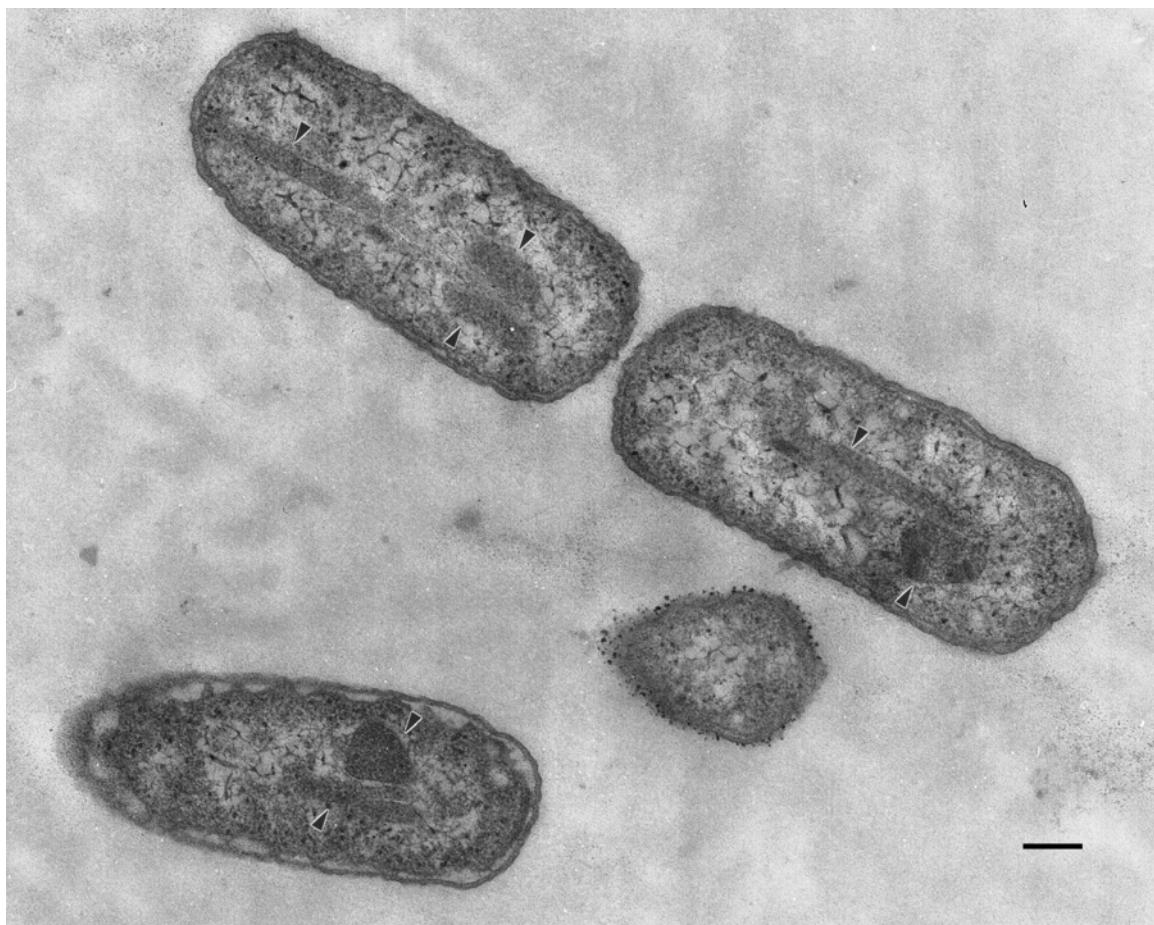


Figure 2-8. Electron micrograph of the aberrant polyhedra formed by *S. enterica pdu* mutant RT818 (*pdu-8::MudJ*). The arrowheads indicate the position of aberrant polyhedral organelles. The bar in the lower right-hand corner is 100 nm in length.

that genes downstream of *pduG* are also involved in the formation of polyhedral organelles. Likely candidates are the *pduJKNTU* genes; the *pduJKT* genes all encode proteins with significant identity to Csos1, a carboxysome shell protein. The *pduN* gene encodes a protein homologous to both CchB and CcmL while the *pduU* gene encodes a protein homologous to EutS. All of these homologues are thought to be involved in carboxysome formation.

Discussion

Previous studies have shown that in addition to being induced by the positive transcriptional regulator, PocR, and its effector, PD, expression of the *pdu* operon is controlled by the CRP/cAMP and ArcA/ArcB global regulatory systems (Andersson 1992, Bobik et al. 1992, Rondon and Escalante-Semerena 1992, Ailion et al. 1993, Chen et al. 1994). Using *lac* fusions it was demonstrated that under aerobic conditions the ArcA/ArcB system had no effect on expression of the *pdu* operon, while the CRP/CAMP system was essential for expression of the *pdu* operon; anaerobically both systems were required for maximal expression (Bobik et al. 1992, Ailion et al. 1993). The results of the studies conducted here on the conditions required for polyhedral organelle formation in *S. enterica* showed that they are analogous to those needed for operon induction; polyhedral organelles were only observed in cells grown either aerobically or anaerobically on poor carbon sources in the presence of PD (Bobik et al. 1992, Ailion et al. 1993). Interestingly, when grown anaerobically on glycerol using fumarate as an electron acceptor, *S. enterica* rarely made polyhedral organelles and when cells with polyhedral organelles were observed they were present in significantly reduced numbers. These growth conditions were previously shown to induce maximal expression of the *pdu* operon (Bobik et al. 1992, Ailion et al. 1993). This indicated that the induction of the

pdu operon alone was not sufficient for polyhedral organelle formation and suggested that PD may play some role in polyhedral organelle formation other than inducing the genes required for formation. Furthermore, results presented here suggest that the *pdu* operon is induced to levels high enough to support polyhedral organelle formation when *S. enterica* grown on poor carbon sources in the presence of PD aerobically; thus, anaerobiosis does not appear to be a prerequisite for polyhedral organelle formation.

A time course of polyhedral organelle formation in *S. enterica* was conducted. Polyhedral organelles were observed in cells shortly after transfer from rich medium to PD/succinate minimal medium suggesting that some aspect of PD metabolism is associated with the polyhedral organelles. Possibly the polyhedral organelles could contribute to a faster growth rate on PD minimal medium since *S. enterica* cells transferred from a mid-log PD minimal culture to fresh PD minimal medium grew at a rate double that of cells transferred from an LB culture. Alternatively, some component of the rich medium carried over to the minimal medium could be inhibiting growth or this phenomenon could simply be the result of induction of the PD degradation pathway enzymes.

TPOP insertion mutations have been used previously to create nonpolar insertion mutations (Way et al. 1984, Rappleye and Roth 1997). The benefit of using these insertion elements is that they are nonpolar when tetracycline is added to the medium and polar when tetracycline is excluded. In this study TPOP mutants were used to study the effect of the *pduABC* genes on the formation of the polyhedral organelles in *S. enterica* cells. Insertion mutations in the *pduA* and *pduB* genes resulted in the absence of polyhedral organelles in cells grown on succinate/PD and PD minimal medium,

respectively. Of the two mutants carrying a TPOP insertion element in the *pduC* gene, strain BE34, was still capable of producing polyhedra. It appeared that this was due to the more distal location of the insertion mutation in the *pduC* gene in strain BE34 as compared to strain BE35, which was unable to form polyhedra. Subsequent tests on strain BE87, containing a precise deletion of the *pduCDE* genes, indicated that the *pduC* gene was not required for the formation of polyhedral organelles substantiating the results seen in strain BE34. Apparently, downstream expression from the TPOP insertion in strain BE35 is not high enough to support polyhedral organelle formation. These results illustrate the difficulties in studying structures in bacteria, which may require strict gene dosage for proper formation.

Complementation studies conducted on strains GH4 (*pduA::TPOP/plac22-pduA*) and GH42 (*pduB::TPOP/plac22-pduB*) to determine if the respective mutations in the *pduA* and *pduB* genes could be complemented using plasmid-encoded sources of the genes suggested that both *pduA* and *pduB* are involved in polyhedral organelle formation since aberrant structures were observed in both strains upon induction of plasmid clones with IPTG. Possible reasons for the inability to correctly form polyhedral organelles could be the requirement of the *pduA* and *pduB* gene products in specific relative amounts, translational coupling of the *pduA* and *pduB* genes, or altered downstream expression from the tetracycline inducible promoters of the TPOP insertions.

Formerly, Orus et al. investigated the biogenesis of carboxysomes using electron microscopy (Orus et al. 1995). They observed what they described as different stages of carboxysome formation. Based on these findings they postulated that the carboxysome shell is made first and RuBisCO is subsequently inserted into the carboxysome through

the shell. During the time course of organelle formation conducted here, no distinct stages of polyhedral organelle formation were noted. In order to examine the packaging of diol dehydratase into the *S. enterica* organelles, complementation studies were conducted on strain BE87 (*ΔpduCDE*, diol dehydratase minus) using a plasmid carrying the diol dehydratase under control of an IPTG inducible promoter. Diol dehydratase was observed to localize to polyhedral organelles in a succinate/PD minimal medium culture that had been induced immediately after transfer from rich medium (prior to polyhedral organelle formation) and also in a mid-log succinate/PD minimal medium culture (subsequent to polyhedral organelle formation). This suggested that diol dehydratase could be packaged into polyhedral organelles after formation. However the possibility that diol dehydratase was incorporated into polyhedral organelles formed during the mid-log phase could not be excluded. Clearly, further study will be needed to clarify this issue. Nevertheless, the fact that plasmid-encoded diol dehydratase could be packaged into the polyhedral organelles could be industrially important providing that the presently unknown function of the polyhedral organelles could provide some advantage to the enzymes that it encased. Future studies to elucidate the mechanism by which diol dehydratase is marked for transport to the polyhedral organelles will be a crucial area to pursue.

The formation of aberrant structures in strain RT818 containing a *Mud* element insertion somewhere downstream of the *pduG* gene demonstrates that genes other than *pduA* and *pduB* are involved polyhedral organelle formation. Possible candidates are the *pduJKNTU* genes, which encode putative homologs to proteins required for the formation of carboxysomes. Although the studies conducted here suggest that the *pduA*, *pduB*, and

other *pdu* genes downstream of *pduG* play some role in the formation of the polyhedra bodies the inability to complement these phenotypes leaves some doubt as to the specific role of these gene products in the synthesis of the polyhedral organelles and illustrates the need for study of additional *pdu* mutants.

CHAPTER 3

PduA IS A SHELL PROTEIN OF POLYHEDRAL ORGANELLES INVOLVED IN THE B₁₂-DEPENDENT DEGRADATION OF 1,2-PROPANEDIOL IN *Salmonella enterica* SEROVAR TYPHIMURIUM LT2

Introduction

Salmonella enterica degrades 1,2-propanediol (PD) in a coenzyme B₁₂-dependent manner (Jeter 1990). 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* (Lawrence and Roth 1996, Price-Carter et al. 2001). In vivo expression technology (IVET) has indicated that PD 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). Moreover, PD degradation by *S. enterica* provides an important model system for understanding coenzyme B₁₂-dependent processes some of which are important in human physiology, industry and the environment (Roth et al. 1996).

At first glance, the degradation of PD appears to be a relatively simple process. The proposed pathway begins with the conversion of PD to propionaldehyde; a process mediated by coenzyme B₁₂-dependent diol dehydratase (Toraya et al. 1979, Obradors et al. 1988). The aldehyde is then converted to propanol and propionic acid presumably by alcohol dehydrogenase, coenzyme A (CoA)-dependent aldehyde dehydrogenase, phosphotransacylase, and propionate kinase. 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 PD occurs aerobically, or anaerobically when tetrathionate is supplied as an electron acceptor (Price-Carter et al. 2001).

The complexity of PD degradation became apparent when the DNA sequence of the *pdu* locus was determined, and twenty-three *pdu* genes were identified: six *pdu* genes are thought to encode enzymes needed for the PD degradative pathway; two are involved in transport and regulation; two are probably involved in diol dehydratase reactivation; one is needed for the conversion of vitamin B₁₂ (CN-B₁₂) to coenzyme B₁₂; five are of unknown function; and seven share similarity to genes involved carboxysome formation (Bobik et al. 1999). Carboxysomes are polyhedral organelles found in cyanobacteria and some chemoautotrophs (Shively et al. 1973b, Shively and English 1991, Shively et al. 1998). 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 CO₂ concentrations and are thought to function as part of a CO₂-concentrating mechanism (Price and Badger 1991, Price et al. 1998, Kaplan and Reinhold 1999).

Recently, *S. enterica* was shown to form polyhedral organelles that resemble carboxysomes during aerobic and anaerobic growth on PD (Bobik et al. 1999). These organelles are approximately 150 nm in cross-section and appear to consist of a proteinaceous shell and interior (Bobik et al. 1999). However, there 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 PD degradation and are associated with coenzyme B₁₂-dependent diol dehydratase and perhaps other enzymes (Bobik et al. 1999). 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 PD 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 (Shively et al. 1998, Bobik et al. 1999). The role of these *pdu* genes in organelle formation have not been investigated, and no genetic study on organelle function has 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 wild type on minimal medium containing lower concentrations of PD, but exhibited a period of "interrupted" growth at higher concentrations which was uncorrectable with high CO₂ supplementation. 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 PD or CN-B₁₂, but are consistent with a role in moderating aldehyde toxicity.

Materials and Methods

Chemicals and Reagents

Formaldehyde (r, s), PD, trichloroacetic acid, and antibiotics were from Sigma Chemical Company (St. Louis, MO). Isopropyl-β-D-thiogalactopyranoside (IPTG) was from Diagnostic Chemicals Limited (Charlottetown, PEI, Canada). Restriction enzymes were from New England Biolabs (Beverly, MA) or Promega (Madison, WI). T4 DNA

ligase was from New England Biolabs. Electrophoresis supplies were from Bio-Rad (Hercules, CA). Bacterial Protein Extraction Reagent II (B-PER II) and Micro BCA reagents were from Pierce (Rockford, IL). 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 3-1](#). The rich medium used was Luria-Bertani (LB) medium (Difco Laboratories, Detroit, MI) (Miller 1972). The minimal media used was the No-carbon-E (NCE) medium (Vogel and Bonner 1956, Berkowitz et al. 1968, Marco and Orus 1993). 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 mg/ml; ampicillin, 100 mg/ml; kanamycin, 50 mg/ml; spectinomycin, 50 mg/ml; and tetracycline, 20 mg/ml. Ampicillin was used at 15 mg/ml in minimal media. Tetracycline was used at 2 mg/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 *placI^qPO-BglII*. MacConkey/PD/vitamin B₁₂ indicator plates and aldehyde indicator plates were prepared as described previously (Conway et al. 1987, Bobik et al. 1999).

General Protein and Molecular Methods

Plasmid purification, bacterial transformation, electrophoresis, and other standard molecular and protein methods were performed as previously described (Sambrook et al. 1989, Johnson et al. 2001).

P22 Transduction and Transposon Mutagenesis of the *pdu* Region

Transductional crosses were performed as described previously (Schmieger 1971, Davis et al. 1980). For TPOP1 mutagenesis of the *pdu* region, a pool of approximately 80,000 independent insertion mutants was prepared as described (Bobik et al. 1992). The pool was then used as a donor in transductional crosses with strain BE235 (*pdu12::MudJ*). Tetracycline resistance was selected, and transductants were screened for loss of the *pdu12::MudJ* elements using MacConkey/lactose/PD 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'-ACCTTGTCACCAACGCTTTCC-3' (forward) and 5'-GTTCATATGCGAAACCACTTC-3' (reverse). 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 base pair 160 of the *pduA* coding sequence.

Growth Curves

Cells were grown in 125 ml baffled Erlenmeyer flasks containing 10 ml of the appropriate medium. The cultures were incubated at 37°C in a New Brunswick model C-24 water bath (Edison, N.J.) with the shaking set at 275 RPM. Cell growth was determined by measuring the optical density at 600nm using a Beckman model DU640 Spectrophotometer. For physiological studies that employed varying CN-B₁₂ and PD concentrations as well as studies examining the effect of CO₂, supplementation cells were

Table 3-1. Bacterial strains

Species	Strain	Genotype
<i>E. coli</i>		
	BE11	(<i>E. coli</i> ER2267) $\text{e}14^-$ (<i>MrcA</i> ⁻) <i>endA1 supE44 thi-1 relA1? RfbD1? SpoT1? } \Delta(mrcC-mrr) 114::IS10 $\Delta(argF-lac)U169 recA1/F' proA^+ B^+ lacI^q$ $\Delta(lacz)M15 zzf::mini-Tn10 (\text{Kan}^r)/\text{pMGS2}$</i>
	BE229	(<i>E. coli</i> ER1992) $F^- \lambda^- \Delta(argF-lac) U169 supE44$ $\text{e}14^- dind1::\text{MudI1473} (\text{Kan}^R, lacZ^+) rtbD1? relA1?$ $endA1 spotT1? thi1 \Delta(mrcL-mrr) 114::IS10/\text{pGH48}$ ($\text{His}_6\text{-PduA}, \text{Ap}^R$)
	BE230	BL21 (DE3)/ pGH48($\text{His}_6\text{-PduA}, \text{Ap}^R$) and pSJS1240(<i>ileX argU, Sp</i> ^r)
	BE231	BL21 (DE3) RIL/pGH107 (T7 expression vector with <i>pduJ</i> insert, Kan^r)
	BL21(DE3)	(<i>E. coli</i> B) $F^- ompT hsdS (r_B-m_B-) dcm^+ T et^r gal \lambda$ (DE3) <i>endA</i>
	BL21(DE3) RIL	(<i>E. coli</i> B) $F^- ompT hsdS (r_B^-m_B^-) dcm^+ \text{Tet}^r gal \lambda$ (DE3) <i>endA</i> Hte/pACYC (<i>argU ileY leuW, Cam</i> ^r)
<i>S. enterica</i> serovar Typhimurium LT2	RT1679	DH5α/pVJ70 (contains <i>pduF'ABCDE ORF1', Ap</i> ^r) Wild type
	BE6	<i>MetE205 ara-9 } \Delta299</i> (in $\Delta299$ the entire <i>cob</i> and <i>pdu</i> operons are deleted)
	BE33	<i>pduA672:: TPOP1</i>
	BE39	<i>pduA673 TPOP1::fragment</i> (a short nonpolar insertion resulting from the spontaneous deletion of the majority of the <i>pduA672::TPOP1</i> element) TR6579/pKD46 ^a
	BE103	$\Delta pduA652$ (precise nonpolar deletion of the <i>pduA</i> gene-see material and methods)
	BE182	$\Delta pduA652/pTA749 (placI^qPO-BglII$ with <i>pduA</i> insert, Ap^r)
	BE228	BE6/ <i>placI^qPO-BglII</i> (no insert, Ap^r)
	BE232	BE6/ <i>placI^qPO-BglII</i> (no insert, Ap^r)
	BE233	BE6/pTA749 (<i>pduA</i> under <i>plac</i> control, Ap^r)
	BE235	<i>pdu12::MudJ</i>
	TR6579	<i>metA22 metE551 trpD2 ivl-452 hsdLT6 hsdSA29</i> HsdB ⁻ strA120 GalE ⁻ Leu ⁻ Pro ⁻

^a(Datsenko and Wanner 2000)

grown in 16x100 mm test tubes containing 5 ml of appropriate medium. Cultures were incubated as described for the aerobic growth except the tubes were held in place at an angle of 45°. Cell growth was monitored by measuring optical density at 600 nm using a Spectronic 20D⁺ spectrophotometer. When cultures were supplemented with CO₂ they were grown in a Forma Scientific CO₂ Water Jacketed Incubator Model 3110 at 37°C and 5% CO₂ on a New Brunswick Gyratory Shaker ModelG2 at 275 RPM.

Inocula for the growth experiments were prepared as follows: bacterial strains were grown overnight at 37°C with shaking at 275 RPM in LB medium; cells from 1.5 ml of an overnight culture were pelleted by centrifugation and resuspended in 1 ml of growth medium, and 0.125 ml was used to inoculate 5 ml cultures or 0.250 ml for 10 ml cultures.

Cloning of *pduA* for High-Level Expression

The following primers were used for PCR amplification of *pduA* DNA using pVJ70 (RT1679) as template: forward, 5'-GGAATTCCATATGCAACAAGCACTAGGAATGG-3' and reverse, 5'-CACCGATGGATCCTCATTGGCTAATTCCCTTCG-3'. The PCR product was gel purified, restricted with *Nde*I and *Bam*HI and ligated to similarly restricted plasmid pET15b. The ligation reaction was heated to 70° C for 15 minutes 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 (Bobik et al. 1999). For protein expression, *E. coli* BL21 (DE3)/pSJS1240 was used as the host strain.

Cloning of *pduJ* for High-Level Expression

The following primers were used for PCR amplification of *pduJ* using pMGS2 (BE11) as template: forward, 5'-GGAATTCCATATGAATAACGCACTGGGACTGG-3'

and reverse, 5'-AGGATCATGCTCGAGGGCTGATTCGGTAAATGG-3'. The PCR product was gel purified, restricted with *Nde*I and *Xho*I and ligated to similarly restricted plasmid pET41a. The ligation reaction was heated to 70° C for 15 minutes and used to transform *E. coli* DH5a by electroporation. The DNA sequence of the cloned DNA was shown to be in agreement with the previously reported *pduJ* DNA sequence (Bobik et al. 1999). 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 l baffled flask with shaking at 275 RPM. When the cells reached an OD₆₀₀ of 0.8, expression of recombinant His₆-PduA protein was induced by addition of 1 mM IPTG. Cells were lysed, and inclusion bodies were isolated by treatment with B-PER 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 through a 0.45 mm pore size cellulose acetate filter. His₆-PduA was purified from the solubilized inclusion bodies using a 1 ml Amersham Pharmacia (Buckinghamshire, England) HiTrap Chelating (Ni²⁺) column. The manufacturer's directions were followed with the following modifications: a 20 ml 20-200 mM linear imidazole gradient was used to elute the recombinant His₆-PduA and 6 M guanidine hydrochloride was added to the binding, wash, and elution buffers.

Antiserum Preparation

His₆-PduA protein obtained from Ni²⁺-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 antisera were prepared in a New Zealand White rabbit by Cocalico Biologicals (Reamstown, PA).

For adsorption of antibodies reacting with PduJ and *E. coli* proteins, two 200 ml cultures of BE231 were prepared using LB/kanamycin/1% glucose medium incubated at 37°C in 1 l baffled flasks with shaking at 275 RPM. When the cells reached an OD₆₀₀ of 0.8, expression of recombinant PduJ-His₈ protein was induced by addition of 1 mM IPTG. An acetone powder made out of whole cells was used to adsorb antibodies reacting with the PduJ protein as described (Harlow and Lane 1988).

Western Blots

Cultures were grown in NCE minimal medium supplemented with 0.4% PD, 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 OD₆₀₀ of 1. Samples were boiled for 8 minutes at 100° C, and 20 µl (equivalent to protein from 0.02 OD₆₀₀ of cells) was separated on a 16.5%Tris-Tricine gel. Electro-blotting was performed in 10 mM MES, 20% MeOH, pH 6.0 using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad), run at 20 V constant for 14.5 hours 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 (Harlow and Lane 1988) using anti-PduA polyclonal antiserum diluted 1:7000 in blocking buffer as the primary antiserum and goat anti-rabbit IgG-AP conjugate (Bio-Rad) diluted 1:3000 in blocking buffer as the secondary antibody. The alkaline color developing reagents, 5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt and p-nitro blue tetrazolium chloride, were used as specified in the manufacturer's directions (Bio-Rad).

Electron Microscopy

For electron microscopy, cells were grown in minimal medium supplemented with 1% succinate and 0.4% PD. 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 (Bobik et al. 1999).

For immunogold localization of the PduA protein and diol dehydratase, cells were sectioned, fixed, observed, and photographed as described previously (Bobik et al. 1999). The primary antisera used were either rabbit polyclonal antiserum against the PduA protein (anti-PduA) diluted 1:100 in PBS, rabbit polyclonal antiserum against diol dehydratase (anti-DDH) diluted 1:1000 in PBS, or preimmune serum diluted 1:100 in PBS. The secondary antibody used was goat anti-rabbit IgG conjugated to 12 nm colloidal gold (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) diluted 1:30 in PBS.

Double-labeling of specimens with both anti-PduA and anti-diol dehydratase polyclonal rabbit antibodies was achieved using a similar protocol with some modifications. Specimens were supported on 400 mesh uncoated “sticky” grids which were made by dipping 400mesh nickel grids into 10 ml of chloroform in which the adhesive from a quarter inch of scotch tape had been dissolved. One side was labeled first with anti-diol dehydratase polyclonal antibody and then with goat-anti-rabbit polyclonal antibody conjugated to 12 nm colloidal gold. The grid was then inverted and the labeling was repeated using anti-PduA polyclonal antibody and goat-anti-rabbit polyclonal antibody conjugated to 18 nm gold.

Construction of a Nonpolar *pduA* Deletion

A nonpolar deletion of *pduA* was constructed as described by Datsenko and Wanner (Datsenko and Wanner 2000) with some modifications. The primers used for PCR amplification of the kanamycin resistance cassette from plasmid pKD4 were 5'-GTGTCCCAACTATCGAACACTCCATGCGAGGTCTTGTAGGCTGGAGCTGC TTCG-3' and 5'-CTGCGCCATGATCTGTTCCACCAGCTCATTGCTGCATGAATATCCTCCTTAGT TC-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'-GTCCTGGCCAGCGCAAGTTCGGC-3', 5'-CAGTCATAGCCGAATAGCCT-3', 5'-CGGTGCCCTGAATGAACTGC-3' and 5'-GCTTTTCCAGCGCATAGCTGGCGCGAGC-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 (Datsenko and Wanner 2000). DNA sequencing showed that the expected deletion was formed; the entire *pduA* coding sequence was deleted except for the last 29 base pairs which included the native ribosome binding site of the *pduB* gene.

DNA Sequencing and Analysis

DNA sequencing was carried out by University of Florida, Interdisciplinary Center for Biotechnology Research, DNA Sequencing Core Facility as described previously (Bobik et al. 1999) and 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, NE). BLAST software was used for sequence similarity searching (Altschul et al. 1990).

Results

Purification of Recombinant His₆-PduA Protein

E. coli strain BE230 was constructed to produce high levels of recombinant His₆-PduA protein. Samples of induced and uninduced boiled cells of this strain were analyzed by SDS-PAGE to determine if the His₆-PduA protein was being expressed. When induced by IPTG, strain BE230 produced high levels of an 11.8 kDa protein ([Figure 3-1, lane 3](#)), which was not seen in the uninduced sample ([Figure 3-1, lane 2](#)). This observed mass correlates well with the predicted mass of 11.4 kDa for the recombinant His₆-PduA protein. Electron microscopy revealed that strain BE230 produced rod-like structures in the cytosol when induced with IPTG ([Figure 3-2A and B](#)) and SDS-PAGE showed that these inclusion bodies ([Figure 3-2C](#)) were composed mainly of the His₆-PduA protein ([Figure 3-1, lane 5](#)). Significant amounts of the His₆-PduA protein were not found in the soluble fraction ([Figure 3-1, lane 4](#)).

The His₆-PduA protein was purified from inclusion bodies by Ni²⁺-chromatography under denaturing conditions. The His₆-PduA protein eluted from the column at approximately 160 to 200 mM imidazole. The fractions containing the His₆-PduA protein were analyzed by SDS-PAGE. A single band at the predicted mass for the His₆-PduA protein was observed as well as three faint bands at higher molecular masses ([Figure 3-1, lane 6](#)). About 600 µg of the partially purified His₆-PduA 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 antiserum production.

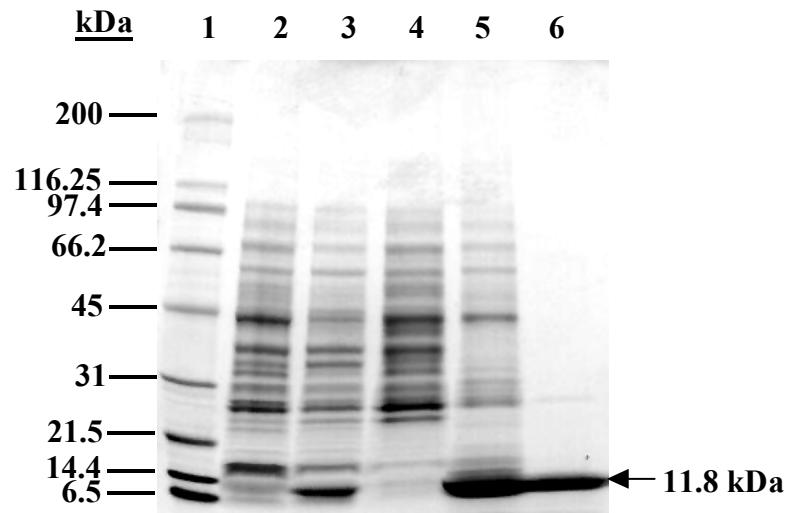


Figure 3-1. Overexpression and purification of the His₆-PduA protein. Lane 1, molecular mass markers; lane 2, uninduced boiled cell lysate; lane 3, induced boiled cell lysate; lane 4, soluble fraction; lane 5, inclusion body prep; lane 6, combined fractions 12 to 15 from Ni²⁺ column elution. Molecular masses in kiloDaltons are shown at the left.

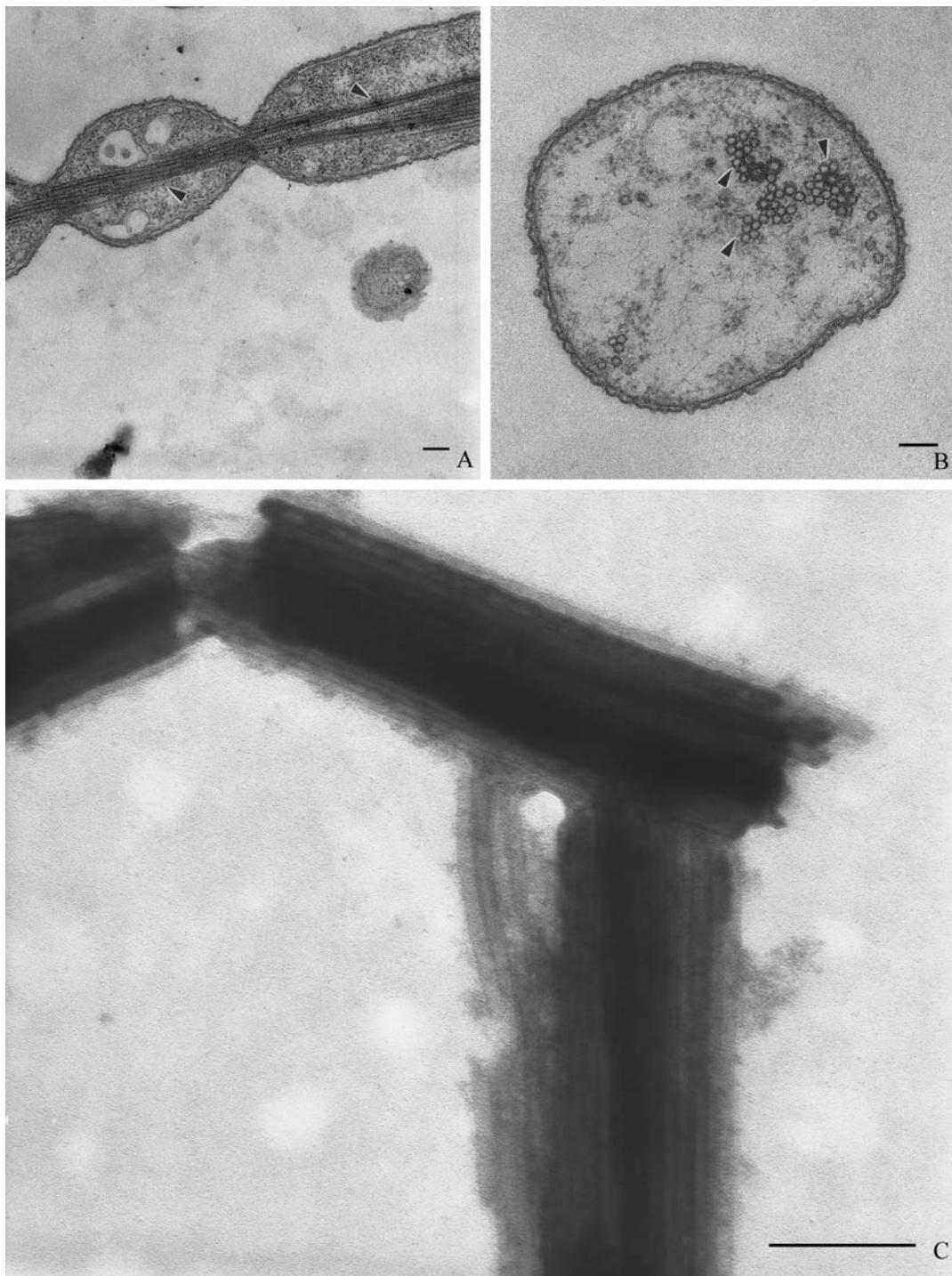


Figure 3-2. Overexpression of the His₆-PduA protein in *E. coli* strain BE230. Cells were grown to mid-log phase and induced with IPTG as described in the text. Plates were purified as described in the materials and methods section. Panel A and B, longitudinal and cross-section of strain BE230 overexpressing the His₆-PduA protein; Panel C, purified PduA plates. Bars in lower right-hand corners are 100 nm in length.

Preparation of PduA-Specific Antiserum

To determine the specificity of the polyclonal antiserum generated against the His₆-PduA protein, western blots were performed on boiled cell lysates. Anti-PduA polyclonal antiserum 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) ([Figure 3-3A, lanes 1 and 2](#)). This indicated that the band at 9.5 kDa corresponded to the native PduA protein. Anti-PduA antiserum also recognized a protein band at 9.5 kDa in strain BE233 ([Figure 3-3A, 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) ([Figure 3-3A, lane 3](#)). These results confirmed that the band at 9.5 kDa was the native PduA protein.

The anti-PduA antiserum preparation also reacted with an 8 kDa protein expressed by the wild type strain and BE182 ([Figure 3-3A, lanes 1 and 2](#)), and a 7.5 kDa protein band expressed by BE233 and BE232 ([Figure 3-3A, lanes 3 and 4](#)). Additional western blots of strain BE6 a *pdu* operon deletion mutant and strain BE231, which overexpresses a PduJ-His₈ fusion protein, indicated that this 8 kDa protein was the PduJ protein ([Figure 3-3C, lane 3](#)). 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 an isogenic strain (BE6) that lacked this plasmid ([Figure 3-3C, lanes 1 and 2](#)). In order to improve specificity of the polyclonal antiserum preparation an adsorption 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 absorb antibodies

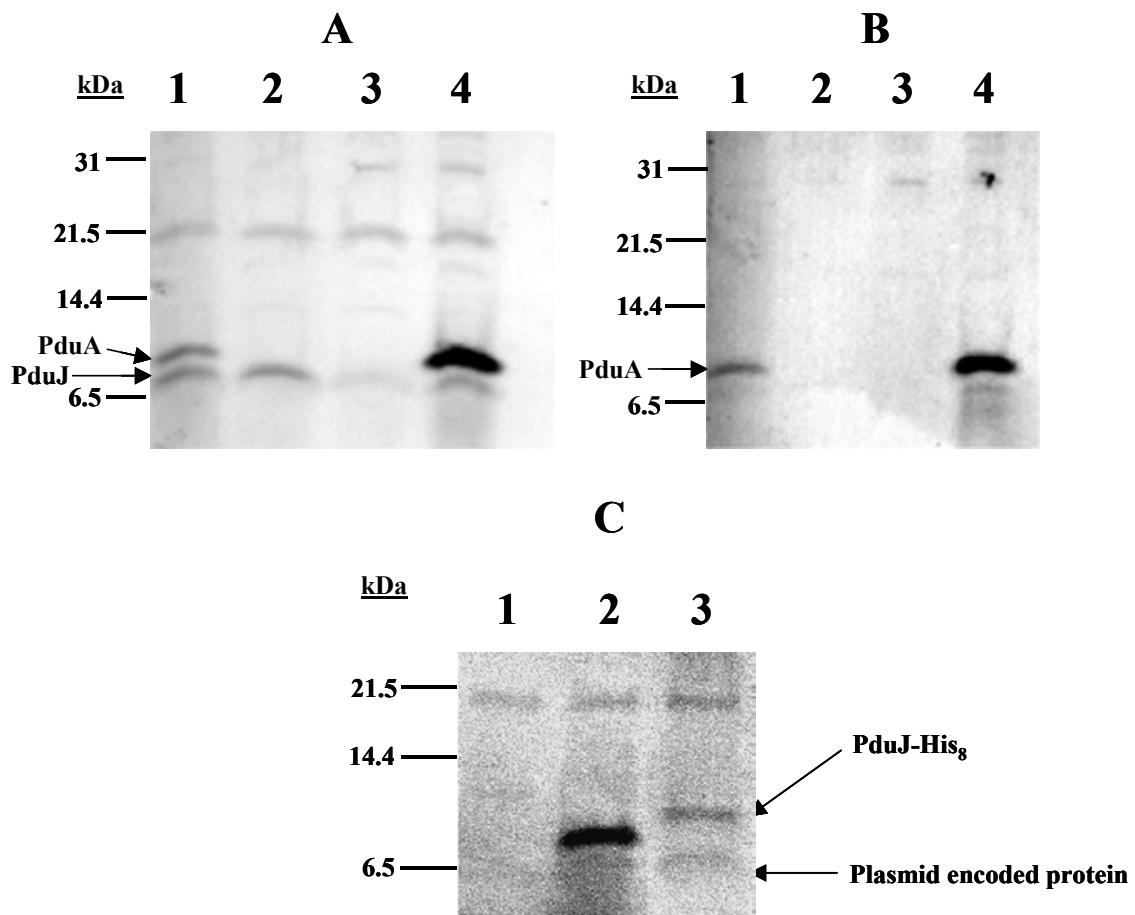


Figure 3-3. Western analysis with untreated and absorbed anti-PduA polyclonal antisera preparations. Panel A and C- untreated. Panel B- absorbed (See Material and Methods section for details). For panels A and B: lane 1, *S. enterica* serovar typhimurium LT2; lane 2, BE182 ($\Delta pduA$ mutant); lane 3, BE232 (isogenic to BE233, except that the expression plasmid lacks an insert); lane 4, BE233 (PduA expression strain). Panel C: lane 1, BE6 (*pdu* operon deleted); lane 2, BE232, (isogenic to BE6 but contains PduA expression plasmid); lane 3, BE231 (PduJ 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 0.02 OD₆₀₀ of cells.

recognizing the PduJ protein and *E. coli* proteins from the anti-PduA polyclonal antiserum preparation. After adsorption, the antiserum preparation recognized a singleband at 9.5 kDa in the wild type strain ([Figure 3-3B, lane 1](#)), and no labeling was observed in the *pduA* deletion mutant, BE182 ([Figure 3-3B, lane 2](#)). The absorbed polyclonal antiserum preparation also recognized a 9.5 kDa protein band in strain BE233 which overexpresses the PduA protein ([Figure 3-3B, lane 4](#)). No labeling at 9.5 kDa was observed in control strain BE232 which carried the plasmid without insert ([Figure 3-3B, lane 3](#)). These results showed that the absorbed anti-PduA antiserum preparation was highly specific for the PduA protein, and this antiserum preparation was used for subsequent immunolabeling experiments.

Localization of PduA by Immunoelectron Microscopy

Immunogold labeling of *S. enterica* cells with highly specific adsorbed anti-PduA antiserum (see above) indicated that the PduA protein is a component of the shell of the polyhedral organelles. In the micrograph shown in [Figure 3-4C](#), 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 ($\Delta pduA$) was labeled with anti-PduA antiserum ([Figure 3-4D](#)).

Localization of Diol Dehydratase in a *pduA* Mutant

Labeling of wild type and BE182 ($\Delta pduA$) cells with anti-diol dehydratase antibodies illustrated the effect of a nonpolar *pduA* deletion mutation on the localization of diol dehydratase ([Figure 3-4A and 3B](#)). In the wild type strain, diol dehydratase

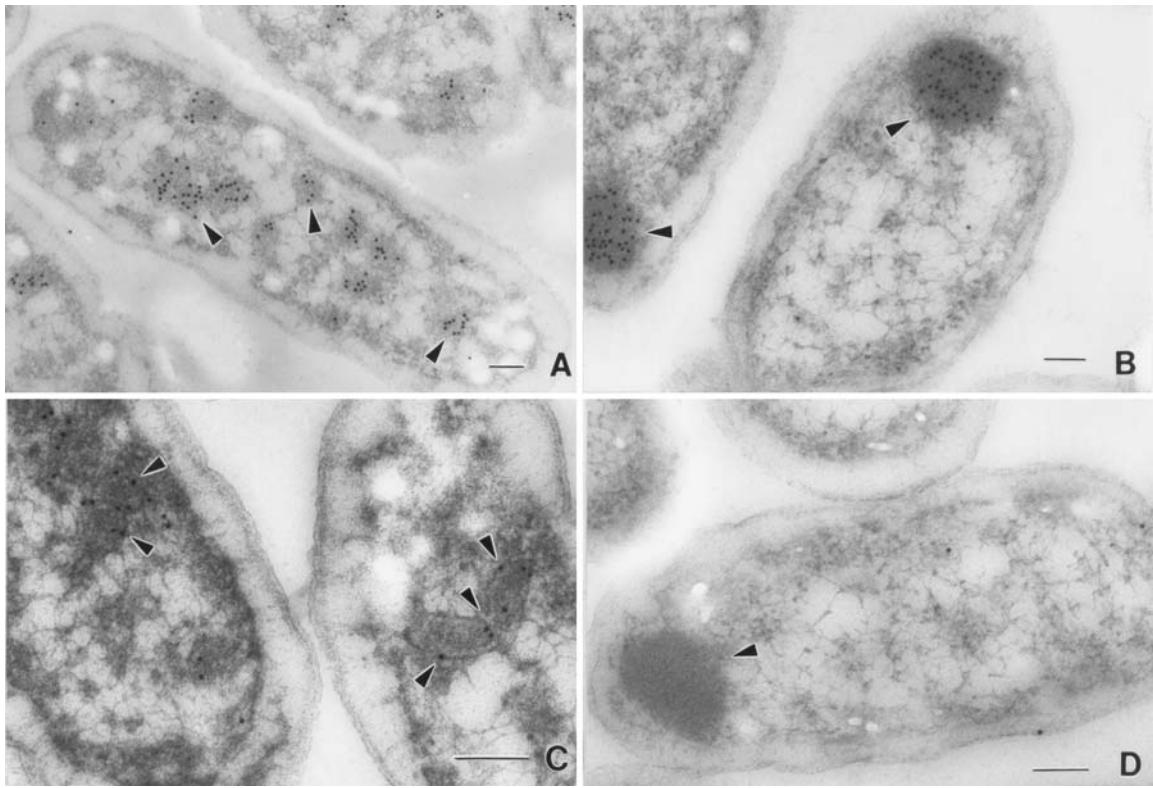


Figure 3-4. Localization of diol dehydratase and the PduA protein by immunoelectron microscopy. Cells were grown on succinate minimal medium supplemented with PD to induce expression of the *pdu* operon. For each panel the arrows point to gold particles that indicate the location of either diol dehydratase or the PduA protein. The strain and protein labeled in each panel are as follows: A, *S. enterica*, diol dehydratase; B, BE182 ($\Delta pduA$), diol dehydratase; C, *S. enterica*, PduA; D, BE182 ($\Delta pduA$), PduA. Bars in lower right-hand corners are 100 nm in length.

localized to the polyhedral organelles ([Figure 3-4A](#)); whereas in strain BE182 ($\Delta pduA$), which is unable to synthesize polyhedra, diol dehydratase was found in large diffuse aggregates at the poles of the cell ([Figure 3-4B](#)). 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 (Rappleye and Roth 1997). Strain BE33 (*pduA*672::TPOP1) exhibited a polyhedra minus phenotype and failed to grow on PD/CN-B12 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 PD minimal medium was restored. This indicated that the *pduA* gene was required for organelle formation, but not for growth on PD. Two additional nonpolar *pduA* mutations were also shown to prevent organelle formation, but allow growth on PD/CN-B₁₂ minimal medium. They were BE182 ($\Delta pduA$) 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 PD.

Co-Localization of the PduA Protein and Diol Dehydratase.

Double-labeling experiments were performed on wild type cells to examine the relative spatial arrangement of the PduA protein and diol dehydratase. When wild type cells were double labeled with anti-diol dehydratase and anti-PduA ([Figure 3-5](#)) both proteins were found to localize to the polyhedral organelles. In [Figure 3-5A](#), the larger

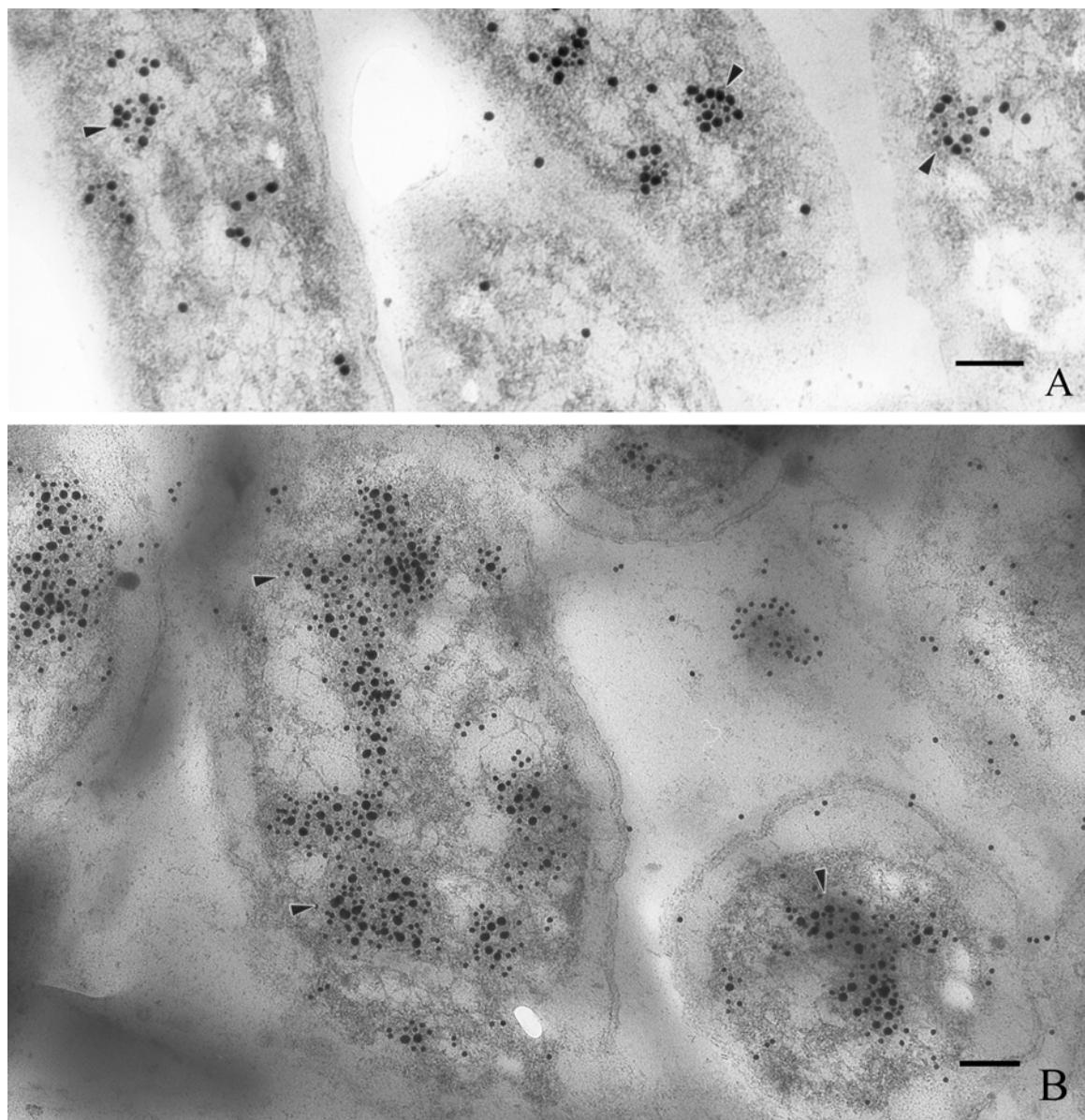


Figure 3-5. Co-localization of diol dehydratase and the PduA protein. Panel A, *S. enterica*, diol dehydratase, 18 nm gold, and PduA, 12 nm gold. Panel B *S. enterica*, diol dehydratase, 12 nm gold, and PduA, 18 nm gold. Bars in lower right-hand corners are 100 nm in length.

(18 nm) gold particles indicate the position of the PduA protein and the smaller (12 nm) gold particles indicate the position of diol dehydratase. From the figure, it is evident that the PduA protein localized to the periphery and diol dehydratase to the interior of the polyhedral organelles. A similar organization was observed when the larger gold particles were used to label diol dehydratase and the smaller gold particles were used to label PduA ([Figure 3-5B](#)). Due to the inherent difficulties associated with this particular procedure not enough cells were collected to determine the statistical significance of this double-labeling experiment. Nevertheless, these findings suggest that diol dehydratase is encased within a shell that is at least partly composed of the PduA protein.

Complementation of the Polyhedral Organelle Minus Phenotype of a *ΔpduA* 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 *ΔpduA* mutation for polyhedral body formation, strain BE228 was grown on PD, 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 ([Figure 3-6A](#)). In the absence of IPTG, no normally shaped organelles were formed and aberrantly shaped structures were rare. The finding that BE228 (*ΔpduA/pTA749*) produced apparently normal organelles in the presence of IPTG, but not in its absence, showed that the organelle minus phenotype of strain BE182 was a consequence of the *ΔpduA* mutation. The apparent explanation for the formation of the 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

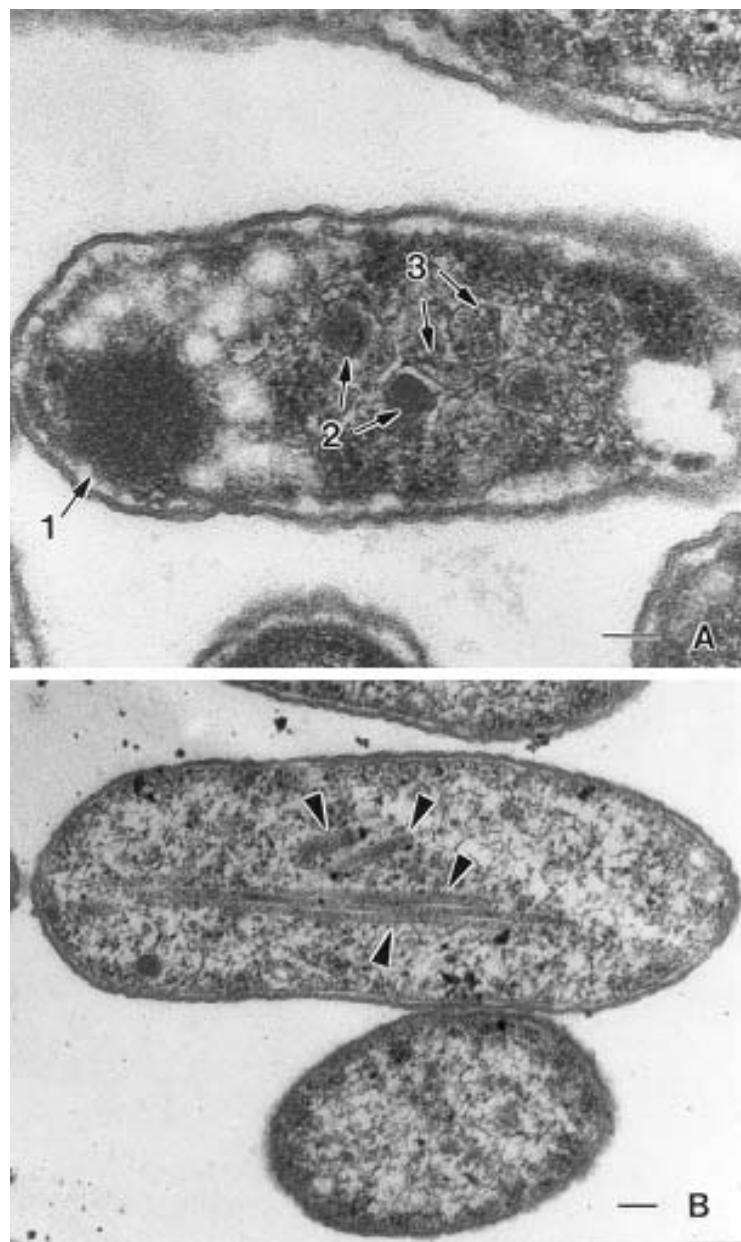


Figure 3-6. Complementation of a Δ *pduA* mutation for formation of polyhedral organelles. Strain BE228 (Δ *pduA*/pTA749- PduA expression plasmid) was grown in minimal succinate medium supplemented with PD and IPTG then examined by transmission electron microscopy. For Panels A and B, strain BE228 was grown with 0.01 and 0.1 mM IPTG, respectively. In Panel A, the numbered arrows indicate the location of the following structures: 1, polar inclusion body; 2, polyhedral organelles; 3, abnormally shaped organelles. In Panel B, the arrowheads indicate the aberrant rod-like structures observed in some cells. Bars in the lower right-hand corners are 100 nm in length.

increasing IPTG concentration (increasing levels of the PduA protein), and at concentrations ≥ 0.1 mM, primarily aberrant structures were observed ([Figure 3-6B](#)). The fact that higher PduA expression levels alter 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 PD/CN-B₁₂ Minimal Medium

During aerobic growth on PD/B₁₂/VILT minimal medium, strain BE182 ($\Delta pduA$) grew similarly to the wild type strain except for a period of “interrupted” growth that initiated at 15 h and persisted until the 40 h mark ([Figure 3-7](#)). Before the onset of “interrupted” growth, BE182 ($\Delta pduA$) grew with a generation time of 6.8 hours which was slightly lower than the 8.4 h generation time of the wild type-strain. Strain BE182 ($\Delta pduA$) also grew faster than wild type after the 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 densities reached by both the wild type strain and BE182 ($\Delta pduA$) were 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 PD. 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, this seems unlikely since “interrupted” growth was dependent on the concentration of PD and CN-B₁₂ present in the growth medium (see below).

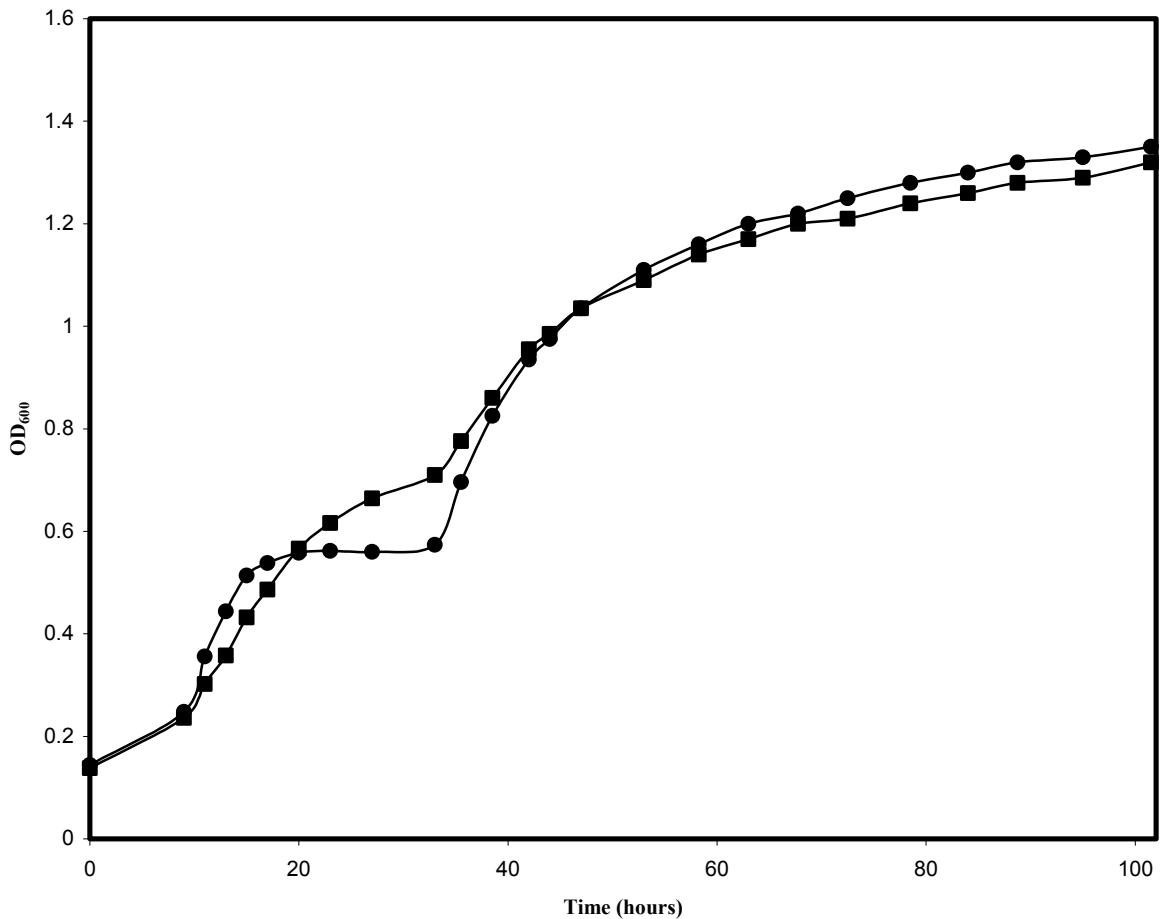


Figure 3-7. Growth of the wild type strain and a *pduA* mutant on PD/CN-B₁₂ minimal medium. Wild-type, *S. enterica* serovar Typhimurium LT2 (■); BE182 (nonpolar $\Delta pduA$) (●). Cells were cultured as described in the Materials and Methods. OD₆₀₀, optical density at 600 nm.

CO₂ Supplementation Does not Correct the “Interrupted” Growth Phenotype of a *pduA* Mutant Strain

In both cyanobacteria and chemoautotrophs it has been demonstrated that mutations in genes required for carboxysome formation result in a high-carbon dioxide requiring (HCR) phenotype (Friedberg et al. 1989, Kaplan et al. 1991, Badger and Price 1992, Marco et al. 1994, English et al. 1995, Ohkawa et al. 1998). To determine if high-CO₂ supplementation would correct the “interrupted” growth phenotype observed in strain BE182 (*ΔpduA*); growth of both the wild type strain and BE182 in the presence of 5% CO₂, with and without amino acid supplementation (VILT) ([Figures 3-8 and 3-9](#)) was examined. Either with or without CO₂, both the wild type strain and BE182 grew similarly except for the aforementioned period of “interrupted” growth observed in BE182 ([Figures 3-8 and 3-9](#)). These results indicate that high CO₂ supplementation appeared to have a general growth stimulation effect in the absence of VILT but did not significantly alter the interrupted growth observed in strain BE182, hence CO₂ does not appear to be involved in the function of the polyhedral organelles.

Effects of PD Concentration on the Growth Rates of Wild Type and *pduA* Mutant Strains

The effects of PD concentration on the growth of the wild type and a strain containing a nonpolar *pduA* deletion mutation (BE182) were examined ([Figure 3-10](#)). At PD 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 (*ΔpduA*) were 11.7 and 10.7 h, respectively. At higher PD concentrations, some distinct differences between the wild type and strain BE182 were noted. At PD concentrations of 0.2% and 0.4%, the wild type strain grew with generation

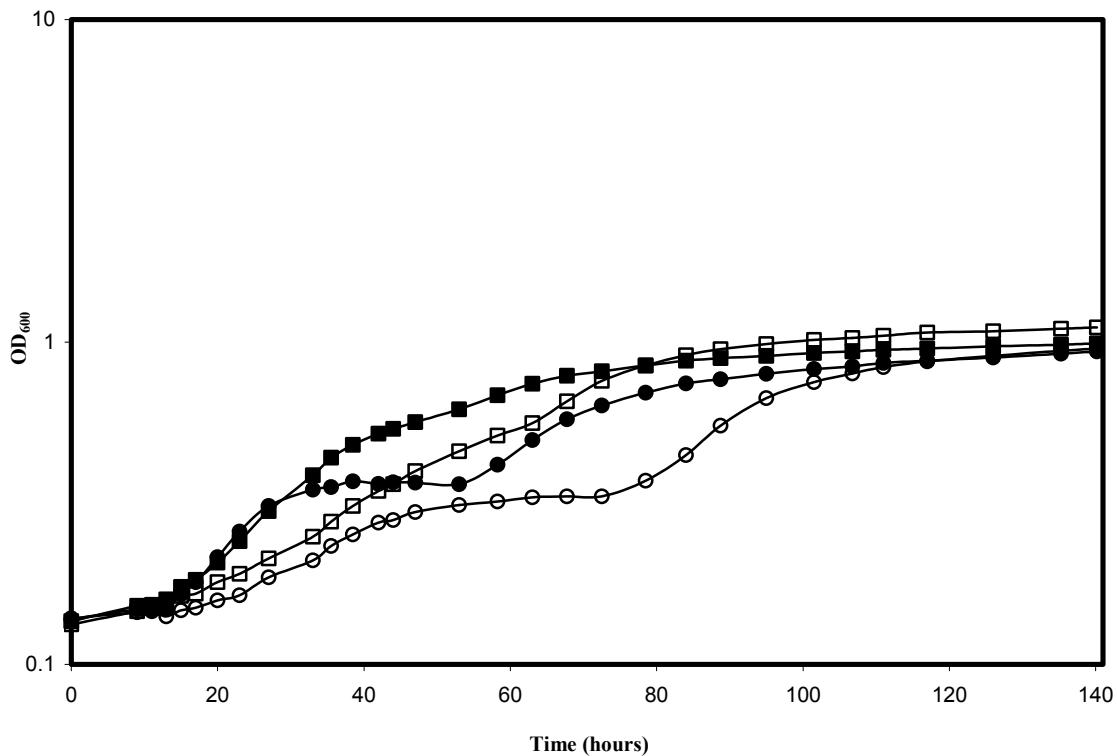


Figure 3-8. The effect of high-CO₂ on growth of the wild type strain and a *pduA* mutant on PD/CN-B₁₂ minimal medium. Cells were cultured on PD/CN-B₁₂ minimal media with (closed symbols) and without 5% CO₂ (open symbols). Wild type, *S. enterica* serovar Typhimurium LT2 (■,□), BE182 (Δ *pduA*) (○,●). OD₆₀₀, optical density at 600nm.

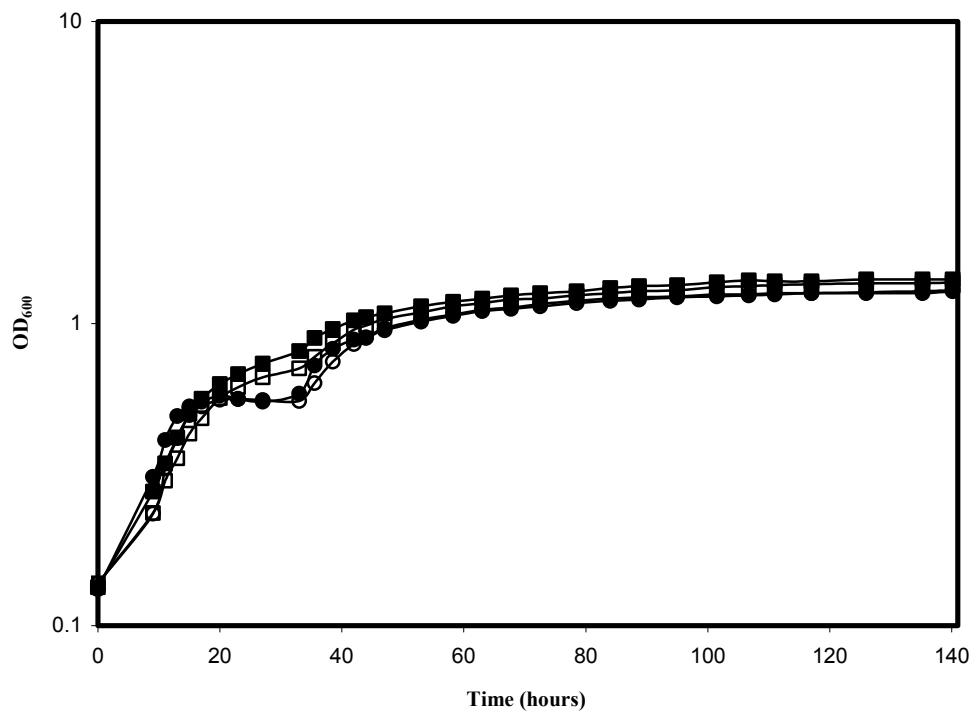


Figure 3-9. The effect of high-CO₂ and VILT supplementation on the growth of strain BE182 (*ΔpduA*) and the wild type strain. Cells were cultured on PD/CN-B₁₂/VILT minimal media with (closed symbols) and without 5% CO₂ (open symbols). Wild type, *S. enterica* serovar Typhimurium LT2 (■,□), BE182 (*ΔpduA*) (○,●). OD₆₀₀, optical density at 600nm.

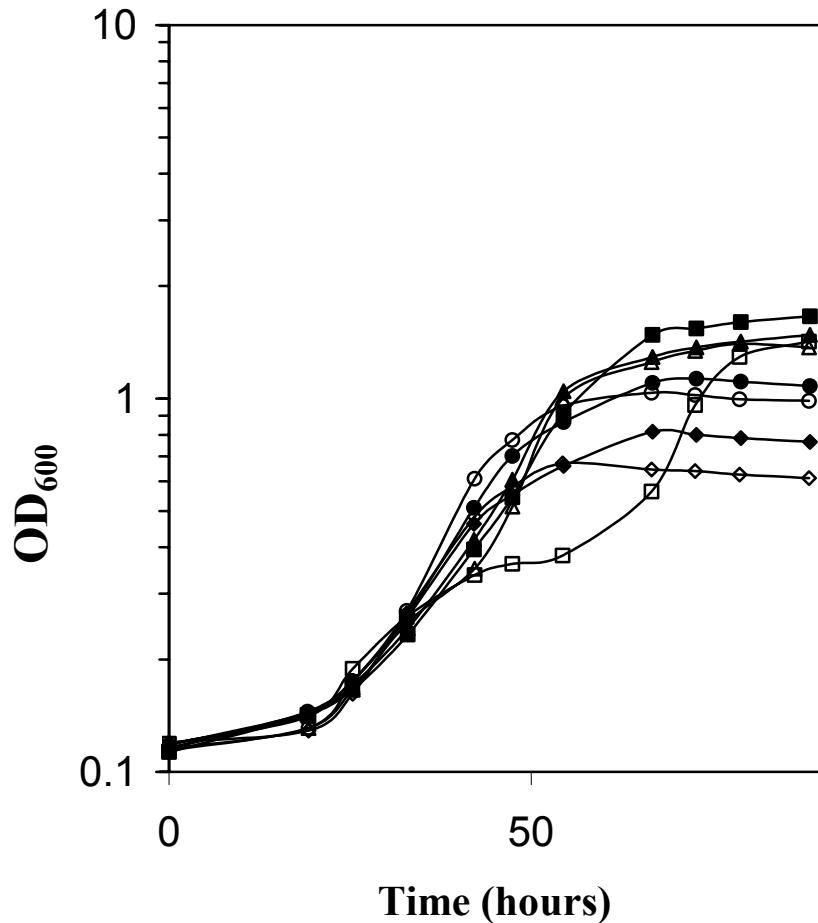


Figure 3-10. The effect of various PD concentrations on the growth of strain BE182 ($\Delta pduA$) and the wild type strain. Cells were cultured on PD/CN-B₁₂ minimal media having the following PD concentrations: 0.4% (■, □), 0.2% (▲, △), 0.1% (●, ○), and 0.05% (◆, ◇). Wild type, *S. enterica* serovar Typhimurium LT2 (closed symbols). BE182 ($\Delta pduA$) (open symbols). OD₆₀₀, optical density at 600nm.

times of 10.2 and 12.6 h. At similar 1,2- PD 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 (*ΔpduA*), the duration of "interrupted" growth was shorter at a PD 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 PD. This suggested that "interrupted" growth resulted from the accumulation of a toxic compound derived from PD. Propionaldehyde was a likely candidate since this compound is an intermediate of PD 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 [Figure 3-10](#). BE182 (*ΔpduA*) grew at a faster rate than the wild type at concentrations of CN-B₁₂ from 0.003125 µg/ml 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 (*ΔpduA*). At CN-B₁₂ concentrations of 0.1 and 0.2 µg/ml, BE182 (*ΔpduA*) 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 PD degradation has a toxic effect that "interrupts" growth. At higher CN-B₁₂ concentrations, PD 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 B₁₂. 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.

Effects of CN-B₁₂ Concentration on the Growth Rates of Wild Type and *pduA* Null Mutant Strains

The effects of CN-B₁₂ concentration on the growth of the wild type and a strain containing a nonpolar *pduA* deletion mutation (BE182) were examined ([Figure 3-11](#)). Strain BE182 (*ΔpduA*) grew at a faster rate than the wild type at concentrations of CN-B₁₂ from 0.003125 µg/ml 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 (*ΔpduA*). At CN-B₁₂ concentrations of 0.1 and 0.2 µg/ml, BE182 (*ΔpduA*) 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 PD degradation has a toxic effect that “interrupts” growth. At higher CN-B₁₂ concentrations, PD 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 B₁₂. 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.

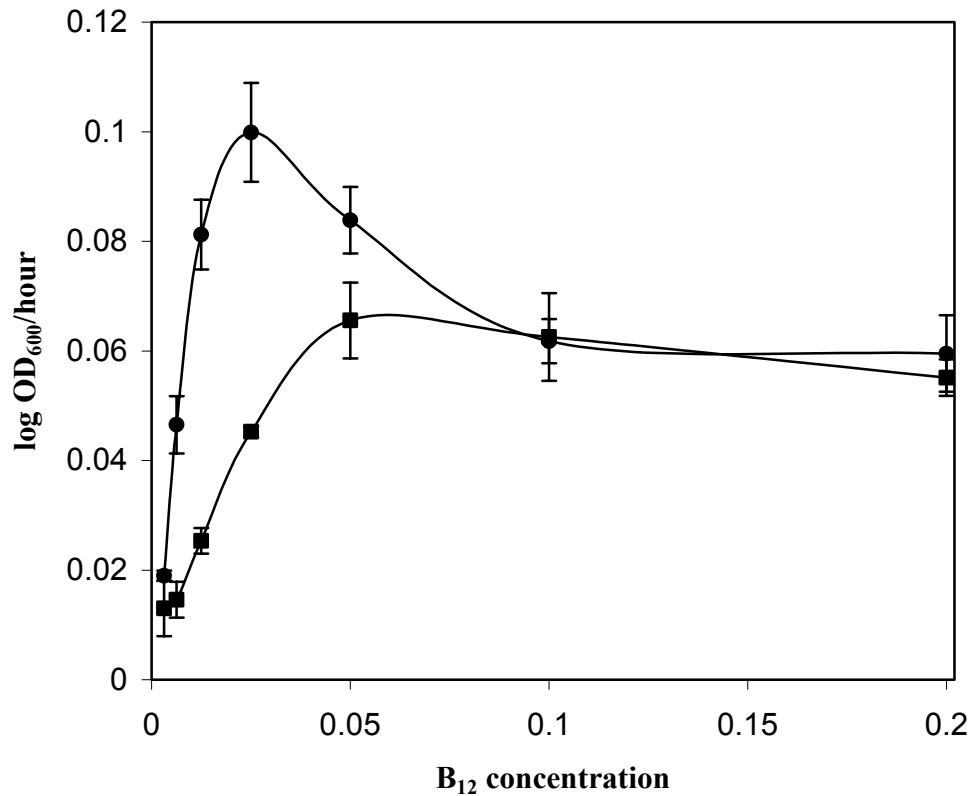


Figure 3-11. The effects of various CN-B₁₂ concentrations on the growth of strain BE182 ($\Delta pduA$) and the wild type strain (*S. enterica*). Cells were cultured on PD minimal medium supplemented with CN-B₁₂ at the concentrations indicated. Wild-type, (■); BE182 ($\Delta pduA$) (●). Error bars represent one standard deviation. OD₆₀₀, optical density at 600nm.

Discussion

Previously, we showed that *S. enterica* formed polyhedral organelles during growth on PD/CN-B₁₂ minimal medium (Bobik et al. 1999). Based on electron microscopy studies and analogy with carboxysomes, we proposed that these organelles consisted of a proteinaceous shell that encased coenzyme B₁₂-dependent diol dehydratase and perhaps other enzymes (Bobik et al. 1999). 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 ([Figure 3-4C](#)). Strains having nonpolar *pduA* mutations were unable to synthesize organelles ([Figure 3-4D](#)), and expression of the PduA protein from a plasmid vector resulted in aberrantly shaped organelles at higher expression levels ([Figure 3-6](#)). 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 of the organelle. A $\Delta pduA$ strain (organelle minus) grew faster than the wild type at limiting B₁₂ concentrations suggesting that the organelles act as barriers to B₁₂. 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 ([Figure 3-4, A and B](#)). Prior immunogold labeling studies showed that diol dehydratase localized primarily to the interior of the organelles (Bobik et al. 1999). Hence, several findings indicate that diol dehydratase localizes within the lumen of the organelles. Although the results of the double labeling experiments suggest that diol

dehydratase is encased with the polyhedral organelles, the possibility that diol dehydratase is also a component of 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 (Bobik et al. 1999). Furthermore, the shells of carboxysomes are known to be composed of multiple proteins (Shively and English 1991, Shively et al. 1998), and the occurrence of multiple shell gene homologues within an operon has been observed previously in *Halothiobacillus* (Baker et al. 1999, Baker et al. 2000), *Synechococcus* (Price et al. 1993, Marco et al. 1994, Ogawa et al. 1994, Ludwig et al. 2000), and *Synechocystis* (Wu Tian et al. 2000).

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 CO₂ for RuBisCO which is encased within the shell of the organelle (Shively and English 1991, Price et al. 1998, Shively et al. 1998, Kaplan and Reinhold 1999). Similarly, the *S. enterica* organelles might be used to concentrate PD (the substrate), or Ado-B₁₂ (the required cofactor) for diol dehydratase. However, the growth studies reported here showed that a *pduA* mutant unable to form organelles grew better than the wild type on lower concentrations of PD and CN-B₁₂ (a precursor of Ado-B₁₂). These results indicated that the *S. enterica* organelles are not involved in the concentration of PD or Ado-B₁₂.

Previously, it was suggested that the polyhedral organelles of *S. enterica* were used to sequester toxic aldehydes (Stojiljkovic et al. 1995). *S. enterica* forms polyhedral organelles during B₁₂-dependent growth on both PD and ethanolamine, and aldehyde intermediates are a common feature of both degradative pathways (Roof and Roth 1988, Jeter 1990). The studies reported here support a model in which the polyhedral organelles of *S. enterica* function to minimize aldehyde toxicity. The finding that *ApduA* mutants undergo a period of "interrupted" growth at higher PD concentrations, but not at lower PD concentrations is consistent with formation of a toxic compound derived from PD. In the first step of PD degradation, coenzyme B₁₂-dependent diol dehydratase catalyzes the formation of propionaldehyde from PD. Presumably, higher concentrations of PD would result in a faster rate of propionaldehyde formation, and greater toxicity. In addition, *pduA* mutants showed "interrupted" growth on PD minimal medium supplemented with higher levels of CN-B₁₂, but not on similar medium supplemented with lower levels of CN-B₁₂. *S. enterica* converts CN-B₁₂ to Ado-B₁₂, the required cofactor for diol dehydratase. Hence, at higher CN-B₁₂ concentrations, it is expected that propionaldehyde would be formed at a faster rate and greater toxicity would result. Following the period of "interrupted" growth, mutants unable to form organelles resumed growth at a rate similar to 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 PD minimal medium supplemented with lower levels of CN-B₁₂. This result indicated that the *S. enterica* organelles present a barrier to Ado-B₁₂. Although this would result in slower growth rates at lower CN-B₁₂

(Ado-B₁₂) concentrations, it could help minimize propionaldehyde toxicity at higher CN-B₁₂ 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-B₁₂ availability.

CHAPTER 4
PURIFICATION AND IDENTIFICATION OF THE MAJOR PROTEINS OF
POLYHEDRAL ORGANELLES INVOLVED IN COENZYME B₁₂-DEPENDENT
DEGRADATION OF 1,2-PROPANEDIOL IN *Salmonella enterica* SEROVAR
TYPHIMURIUM LT2

Introduction

The vitamin B₁₂ coenzymes, adenosyl-B₁₂ (Ado-B₁₂) and methyl-B₁₂ (CH₃-B₁₂) are required cofactors for at least 15 different enzymes (Schneider and Stroinski 1987a, Roth et al. 1996). These enzymes have a broad but uneven distribution among living forms and are vital to human health, essential to the carbon cycle, and have important industrial applications (Roth et al. 1996). Historically, bacteria have provided excellent model systems for studying vitamins, and recent investigations with several bacterial systems have found the molecular biology of B₁₂-dependent processes to be unexpectedly complex (Stojiljkovic et al. 1995, Roth et al. 1996, Bobik et al. 1999, Sauvageot et al. 2002). One of the most surprising findings in this area has been the identification of a polyhedral organelle involved in coenzyme B₁₂-dependent 1,2-propanediol (PD) degradation by *Salmonella enterica* (Bobik et al. 1999).

S. enterica utilizes PD as a carbon and energy source in an Ado-B₁₂-dependent fashion (Jeter and Roth 1987). Degradation occurs aerobically, or anaerobically if tetrathionate is added as a terminal electron acceptor (Price-Carter et al. 2001). The ability to degrade PD appears to be important to the lifestyle of *S. enterica* which, including the genes necessary for de novo cofactor synthesis, maintains 40 to 50 genes in order to accomplish this process. Based on biochemical studies, a pathway for PD

degradation has been proposed (Toraya et al. 1979, Obradors et al. 1988). Breakdown initiates with the conversion of PD to propionaldehyde by Ado-B₁₂-dependent PD dehydratase (Abeles and Lee Jr. 1961), a mildly toxic byproduct. The propionaldehyde is then reduced to propanol, or oxidized to propionic acid via propionyl-CoA and propionyl-phosphate. Reduction of propionaldehyde serves to regenerate NAD from NADH, while its oxidation provides a source of ATP and cell carbon.

Because the pathway of PD degradation appeared relatively straight-forward, it was somewhat surprising when DNA sequence analyses indicated that the PD utilization (*pdu*) locus included 23 genes (Bobik et al. 1999). Of these, six *pdu* genes are thought encode enzymes needed for the PD degradative pathway (*pduCDEPQW*); two are involved in transport and regulation (*pduF* and *pocR*); two are probably used for diol dehydratase reactivation (*pduGH*); one is needed for the conversion of CN-B₁₂ to Ado-B₁₂ (*pduO*); five are of unknown function (*pduLMSVX*); and seven (*pduABJKNTU*) share similarity to genes needed for the formation carboxysomes, polyhedral organelles involved in autotrophic CO₂ fixation (Shively and English 1991, Shively et al. 1998, Bobik et al. 1999, Cannon et al. 2002).

The finding that the *pdu* locus included several homologues of carboxysome genes led to recent studies that showed *S. enterica* forms polyhedral organelles the during Ado-B₁₂-dependent growth on PD. (Bobik et al. 1999). Like the carboxysomes, the *pdu* organelles are 100-150 nm in diameter and are composed of a proteinaceous interior surrounded by a 3-4 nm protein shell (Bobik et al. 1999, Havemann et al. 2002). However, carboxysomes and the *S. enterica* organelles differ in a number of ways. Carboxysomes function to enhance autotrophic growth at low CO₂ concentrations (Price

et al. 1998, Shively et al. 1998, Kaplan and Reinhold 1999, Badger and Price 2003), and the carboxysomes of *Halothiobacillus neapolitanus* (which are the best studied) consist of a protein shell composed of at least six different proteins which encases most of the cell's ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) (Cannon and Shively 1983, Holthuijzen et al. 1986, English et al. 1994, Baker et al. 1999, Baker et al. 2000). In contrast, the organelles of *S. enterica* do not contain RuBisCO, but instead consist of Ado-B₁₂-dependent diol dehydratase and a protein shell composed in part of the PduA protein and other unidentified proteins (Bobik et al. 1999, Havemann et al. 2002). It has been proposed that the *S. enterica* organelles function to minimize aldehyde toxicity by moderating propionaldehyde production through control of Ado-B₁₂ availability. However, this function has not been established. Moreover, the mechanism of the *S. enterica* organelles has not been investigated and a great deal remains to be learned about their structure.

Here we report the purification and structural characterization of the unusual organelles involved in PD degradation by *S. enterica*. The analyses performed included one and two-dimensional electrophoresis, immunoblotting, N-terminal sequencing and protein mass fingerprinting via Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS). By these methods, 15 proteins of the organelles were identified. These included Ado-B₁₂-dependent diol dehydratase (PduCDE), CoA-dependent propionaldehyde dehydrogenase (PduP), adenosyltransferase (PduO), the large (PduG) and small (PduH) subunits of the putative diol dehydratase-reactivating factor, the PduA shell protein and six additional, probable structural proteins (*pduBB'JKTU*) as well as one unidentified protein. These findings are consistent with a

role for the *pdu* organelles in aldehyde detoxification and also show that these organelles represent a complex mode of subcellular organization.

Materials and Methods

Chemicals and Reagents

PD was from Sigma Chemical Company (St. Louis, MO), Bacterial Protein Extraction Reagent II (B-PER II) was from Pierce (Rockford, IL), and Pefabloc SC was from Pentapharm Ltd. (Basel, Switzerland). Electrophoresis supplies were from Bio-Rad (Hercules, CA) unless otherwise stated. Other chemicals were from Fisher Scientific (Pittsburgh, PA).

Organelle Purification

For organelle purification, *S. enterica* serovar Typhimurium LT2 was grown in 2.8 l Fernbach flasks containing 1 l of NCE minimal medium (Vogel and Bonner 1956, Berkowitz et al. 1968) supplemented with 1% succinate and 0.4% PD. The inoculum was a 5 ml Luria-Bertani (LB) broth culture and incubation was at 37°C with shaking at 275 RPM. Under these conditions, growth was supported by succinate, but the PD was not metabolized assuring continued high induction of the *pdu* operon (Chen et al. 1995, Bobik et al. 1997). After cultures reached late log phase (OD₆₀₀ between 1 and 1.2), cells from 2 l of media were harvested by centrifugation at 4,000 x g for 10 min. at room temperature. The pelleted cells (5-6 grams) were washed with 300 ml of lysozyme buffer (50 mM Tris-HCl, 0.6 M sucrose, 5 mM EDTA, 0.2% PD, pH 8.0), resuspended in 30 ml of similar buffer containing 2 mg/ml lysozyme, and incubated at 37°C for 2 hr with occasional agitation. Subsequent to digestion with lysozyme all steps were performed at 0-4°C. Cells were pelleted by centrifugation (7,740 x g, 15 min.), washed with lysozyme

buffer and resuspended in sonication buffer (50 mM Tris-HCl, 2 mM EDTA, and 0.2% PD, pH 8.0) at a concentration of 0.1 gram of wet cell mass per ml. Cells were then broken by sonication (four 30 sec. bursts with 1 min. cooling intervals on ice) using a VirSonic 300 sonicator (The Virtis Company, Inc., Gardiner, NY) with a 10 mm diameter disruptor horn and an output setting of 10. After sonication, the crude cell extract was diluted with an equal amount of B-PER II supplemented with 400 mM NaCl and 20 mM MgCl₂ and then incubated at 4°C for 30 minutes on a rotary shaker set at 18 RPM. Unlysed cells and cell debris were removed by centrifugation at 12,000 x g for 10 min. and the resulting supernatant was then subjected to high-speed centrifugation (Beckman SW-27 rotor, 49,000 x g, 90 min.). The pellet was resuspended in 5 ml of TEMP buffer (50 mM Tris-HCl, 1 mM EDTA, 10 mM MgCl₂, 0.2% PD, pH 8.0) and then clarified by low-speed centrifugation (12,000 x g, 10 min.). The clarified preparation was layered over two 35 ml, 35-65%, continuous sucrose gradients in 38.5 ml tubes and centrifuged for 12 hr (Beckman SW-27 rotor, 104,000 x g). The polyhedral organelles formed a white translucent band about 2/3 of the way down the centrifuge tube whereas membrane fragments and amorphous debris formed an opaque tan band below. The fraction containing the polyhedra was collected from each gradient with a plastic pipette, diluted to 38.5 ml with TEMP buffer, and centrifuged at 52,000 x g for 90 min. The pellets were resuspended in 1 ml of TEMP buffer and clarified by centrifugation for 10 min at 4,000 x g using an Eppendorf 5415C microcentrifuge. The supernatant containing the purified organelles was carefully removed and stored at 4°C prior to analysis.

General Protein and Molecular Methods

Protein concentration was determined using the Bio-Rad protein assay and bovine serum albumin (BSA) as the standard. Other molecular and protein methods were performed as previously described (Sambrook et al. 1989).

Electron Microscopy

Samples of polyhedral organelles were placed on 300 or 400 mesh Formvar-coated copper grids, fixed with an equal amount of Trump's reagent (McDowell and Trump 1976) for 5 min., washed with distilled deionized H₂O and then stained with 1% uranyl acetate. Samples were viewed and photographed with a Zeiss EM-10CA transmission electron microscope.

Western Blots

Purified polyhedral organelles (20 µg) were concentrated using a Vivaspin centrifugal filtration device (Sartorius AG; Goettingen, Germany) with a 5,000 molecular mass cut-off and then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 4-20% gradient gel (Bio-Rad). After electrophoresis, the proteins were transferred to a Hybond P membrane (Amersham Biosciences Corp; Piscataway, NJ) and probed as described previously (Havemann et al. 2002) using primary antisera at the following dilutions: anti-PduAJ, 1:3500; anti-diol dehydratase, 1:1000; anti-PduA, 1:3500; anti-PduO, 1:1000; and anti-PduP, 1:1000.

Enzyme Assays

Diol dehydratase assays were performed using the 3-methyl-2-benzothiazolinone hydrazone method as previously described (Toraya et al. 1977). One unit activity is defined as the amount of enzyme that catalyzes the formation of 1 µmol of propionaldehyde per min per mg protein.

Propionaldehyde dehydrogenase activity was followed spectrophotometrically using an NAD linked assay. The volume of each assay was 1 ml and the assay mixture contained 50 mM CHES buffer pH 9.5, 1 mM DTT, 75 nmol NAD⁺, 100 nmol lithium coenzyme A, and the sample of interest. The reaction was initiated by adding 10 µl of 1 M propionaldehyde. Activities were calculated from the initial rate and one unit activity was defined as the amount of enzyme that catalyzes the formation of 1 nmol of NADH per min per mg.

ATP:cob(I)alamin adenosyltransferase assays were performed as previously described (Johnson et al. 2001). One unit of ATP:cob(I)alamin adenosyltransferase activity was defined as 1 nmol of Ado-B₁₂ formed per min per mg protein.

Glycoprotein Staining

To test for the presence of glycosylated proteins, Pierce glycosylation stain was used according to the manufacturer's instructions.

N-terminal Sequencing

Prior to N-terminal sequencing, organelle proteins were separated by 2-dimensional (2D) electrophoresis that employed isoelectric focusing and SDS-PAGE (2D-IEF-SDS-PAGE). Purified polyhedral organelles (230 µg) were concentrated to a 20 µl volume, using a Vivaspin filtration device (Sartorius AG) with a 5,000 molecular mass cut-off, and mixed with 150 µl of rehydration solution: 7 M urea, 2 M thiourea, 4% CHAPS, 0.1% SDS, 65 mM dithiothreitol, 1% immobilized pH gradient (IPG) buffer, pH 3 to 10 (Amersham Biosciences Corp). This sample was then used to rehydrate a 7 cm nonlinear pH 3 to 10 IPG strip (Amersham Biosciences Corp) and isoelectric focusing was performed at 19° C, and 3500 V, for 63 Vhr. Subsequent to isoelectric focusing, the IPG

strip was equilibrated with 50 mM Tris-HCl pH 6.8, 6 M Urea, 30% (v/v) glycerol, 2% (w/v) SDS, 2.5 % iodoacetamide, and a trace amount of bromophenol blue. The strip was then sealed on top of a 10 x 10 cm, 4-20% Tris-glycine gel (Invitrogen; Carlsbad, CA) with warm 0.5% agarose made in 25 mM Tris pH 8.3, 192 mM Glycine, and 0.1% SDS. The gel was run for 20 minutes at 20 V to load the sample and then an additional 90 min at 125 V to resolve the organelle proteins. After electrophoresis, the organelle proteins were transferred to an Immobilon P membrane (Millipore; Billerica, MA) by trans-blotting (14.5 hrs at 20 V and 4°C) and stained with Coomassie brilliant blue R-250. N-terminal sequencing of the organelle proteins separated by 2D-PAGE was performed by the University of Florida, Interdisciplinary Center for Biotechnology Research, Protein Chemistry Core Facility using an Applied Biosystems model 494 HT sequencer and standard blot cartridge cycles.

Matrix Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry (MALDI-TOF MS)

The protein components of purified polyhedral organelles (115 µg) were separated by 2D-IEF-SDS-PAGE as described above, but with the following modifications. The IEF dimension employed a 17 cm linear pH 4 to 7 IPG strip (Bio-Rad) and was focused for a total of 95 kV hours. After the IEF dimension, the IPG strip was sealed onto a 18.3 x19.3 cm, 8-16% polyacrylamide gel (Bio-Rad) and run for 20 minutes at 10 mA to load the sample and then another 5 to 6 hrs at 24 mA to resolve the organelle proteins. Following 2D-electrophoresis, the gel was stained with colloidal blue (Genomic Solutions, Ann Arbor, MI) and organelle proteins were excised, washed, dried, digested with trypsin, purified using a Millipore ZipTip, and then analyzed by MALDI-TOF-MS.

For Mass Spectrometry, a calibration mixture of Angiotensin I, ACTH (clip 1-17), and ACTH (clip 18-39) (Applied Biosystems, Foster City, CA) was prepared according to the manufacturer instructions. It was combined with the sample as an internal standard at a concentration of 1 pmol/ μ L each protein standard. Tryptic digests were co-crystallized with a matrix of α -cyano-4-hydroxycinnamic acid (Aldrich, St. Louis, MO) and analyzed using an Applied Biosystems Voyager-DE Pro MALDI-TOF mass spectrometer operated in the delayed-extraction, reflector mode. This instrument was equipped with a nitrogen laser delivering pulses of ultraviolet light (337 nm) and spectra from 100 individual laser shots were collected for each sample. An accelerating voltage of 20 kV, grid voltage of 72%, and extraction delay time of 200 nsec were used.

Peptide Mass Fingerprinting

Two Web-based programs dedicated to mass-fingerprinting were used to analyze the resulting MALDI-TOF spectra, MS-Fit (<http://prospector.ucsf.edu>) and Profound (http://129.85.19.192/profound_bin/ProFound.exe). The obtained peptide mass fingerprint spectra were analyzed by searching the National Center for Biotechnology Information nonredundant (NCBI-nr) protein database with Profound or one or more of the following databases with MS-Fit: Swiss-Prot, Genbank (Genpept), and NCBInr. The following standard parameters were used with both programs: charge state: MH^+ , protein mass range: 1-100 kDa, all species allowed, full range of pI, one missed cleavage allowed, possible modification of cysteine by acrylamide or carbamidomethylation, possible modification of methionine by oxidation, and peptide mass tolerance of ± 50 ppm. Possible adjustments to the above parameters included: protein mass range and pI range—narrowed or extended according to the 2D gel information, *Salmonella*

typhimurium species was selected, if allowable, and the missed cleavages allowed was increased if larger peptides were present in the spectra. The number of tryptic peptides included in the search were determined as follows: the peptide masses from the spectra were deisotoped and ordered according to peak intensity, the threshold for peak detection was set at 2% Base Peak Intensity to filter out noise and then the entire list was uploaded into the MS-Fit or Profound search program. Calibration standards, trypsin autoproteolysis peptides, and possibly known keratin peaks were placed on an exclusion list and not included in the database search. Possible adjustments to the peptide list included the use of fewer peptides if review of raw spectra showed very few peptides above the baseline noise and/or a second search listing only the most intense peaks (top 5, 10, 20, etc.).

Densitometry Analysis

Organelle proteins were resolved by 2D-IEF-SDS-PAGE as described above under "N-terminal sequencing" with the following changes. The organelle preparation used contained 17 µg protein. The IEF buffer contained a lower concentration of CHAPS (2%) and was supplemented with 1% dodecyl-maltoside. Following 2D-electrophoresis, staining was done with Bio-Rad Sypro Ruby Protein Gel Stain, and the relative amounts of organelle proteins were quantitated using an Alpha Innotech IS-1000 Digital Imaging System (Alpha Innotech Corporation, San Leandro, CA).

Results

Purification of Polyhedral Organelles

The polyhedral organelles involved in PD degradation were purified by a combination of detergent treatment, and differential and density gradient centrifugation.

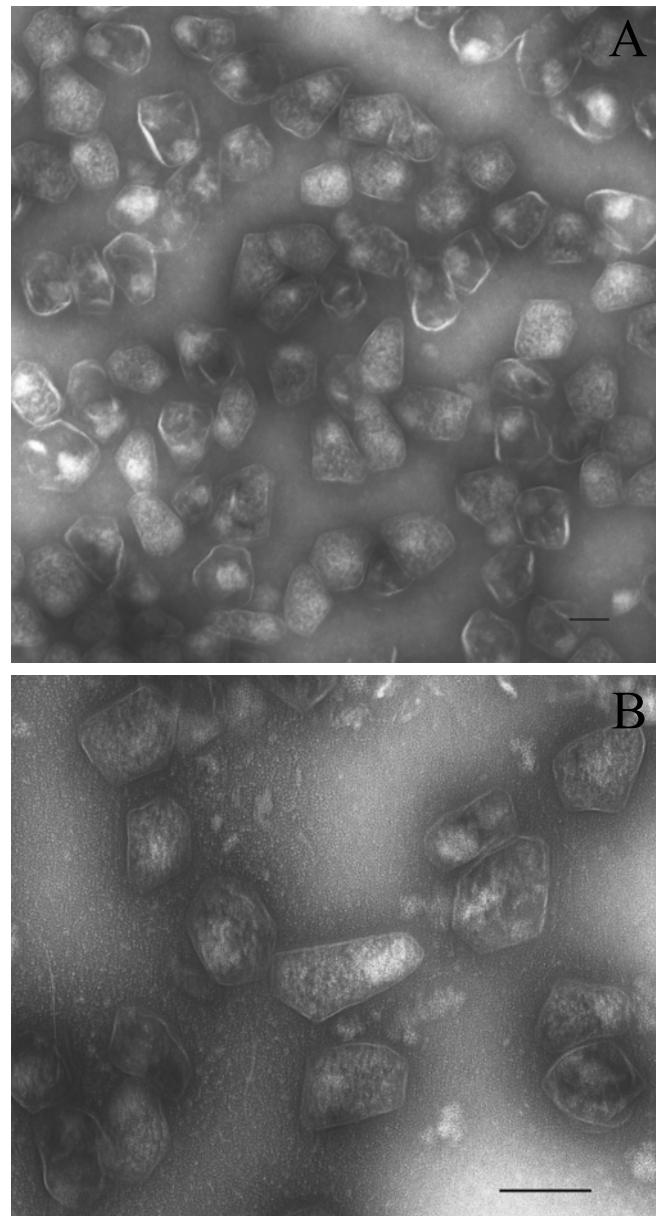


Figure 4-1. Electron micrographs of polyhedral organelles purified from *S. enterica*. A. Low-magnification (53,000X) electron micrograph of polyhedral organelles. B. High-magnification 122,000X) micrograph of polyhedral organelles. The polyhedra are approximately 100-150 nm in diameter and are composed of an interior filled with heterogeneous particles surrounded by a 3-4 nm thick protein shell. The bars in the lower right-hand corners are 100 nm in length. Prior to electron microscopy, organelles were fixed with Trump's reagent and then negatively stained with uranyl-acetate.

The progress of the purification was followed by electron microscopy ([Figure 4-1A and B](#)), SDS-PAGE ([Figure 4-2](#)), and assay of B₁₂-dependent diol dehydratase ([Table 4-1](#)), an enzyme previously shown to be associated with these organelles (Bobik et al. 1999, Havemann et al. 2002).

Electron Microscopy

Electron microscopy showed that the lysis by sonication effectively released many polyhedral organelles. Prolonged treatment (30 min.) of cells with lysozyme and EDTA prior to sonication produced extracts in which polyhedra were better separated from membrane fragments and amorphous debris. Sonication gave a higher yield of purified polyhedra than did lysis of cells using the French press. In crude lysates generated by sonication, polyhedra appeared to be bound to the outside of membrane vesicles whereas in lysates generated using a French Press, they were encased within membrane vesicles, which apparently interfered with purification. In addition, it was important to exclude MgCl₂ from the lysis buffer otherwise the polyhedral organelles pelleted with the unbroken cells and cell debris in the initial centrifugation step.

After sonication and removal of cell debris by centrifugation, soluble extracts were treated with the proprietary detergent, B-PER II, sodium chloride, and magnesium chloride. This removed the majority of lipid vesicles and separated the polyhedra from the remaining vesicles and glycogen rosettes. The subsequent 48,000 x g centrifugation yielded a pellet highly enriched in polyhedra and separated from soluble proteins, while the final low-speed and sucrose density gradient centrifugation steps removed the remaining high molecular weight contaminants.

Electron microscopy indicated that the purified organelles were nearly homogenous

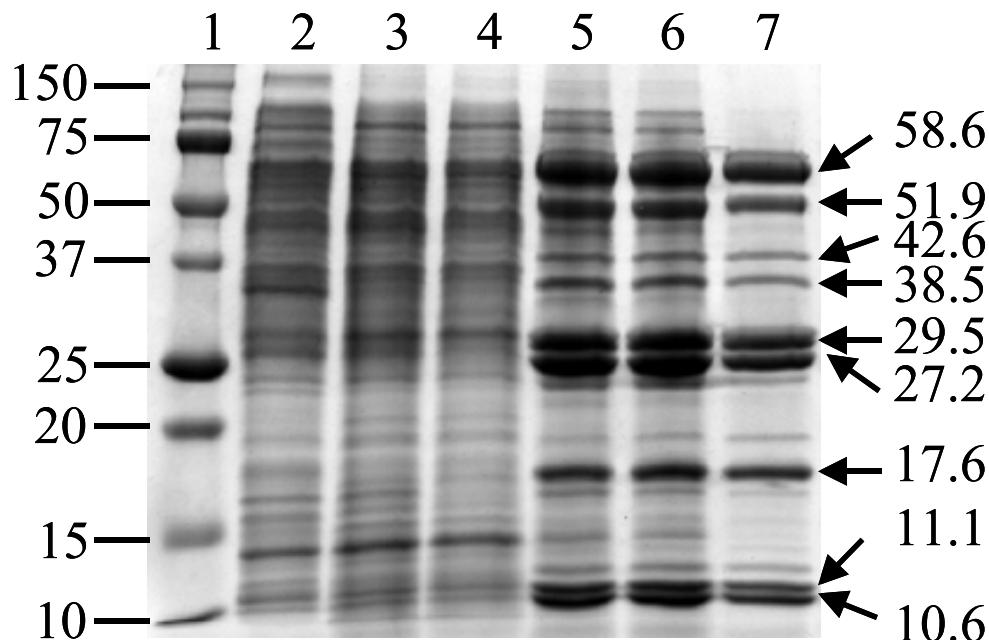


Figure 4-2. Purification of the polyhedral organelles of *S. enterica*. Lanes 2-7 contain 20 µg of protein. Lane 1, molecular mass markers; lane 2, crude lysate; lane 3, detergent-treated crude lysate; lane 4, 12,000 \times g supernatant; lane 5, 49,000 \times g pellet; lane 6, second 12,000 \times g supernatant; lane 7, density gradient purified polyhedral organelles. Molecular masses in kilodaltons are shown at the left.

(Figure 4-1A). Purified preparations contained many polyhedra, but no cells, membrane vesicles, or glycogen rosettes were observed. The polyhedral organelles ranged in size from 100-150 nm in diameter and had a 3-4 nm thick shell surrounding a heterogeneous interior (Figure 4-1B). The general appearance of the polyhedra remained unchanged throughout the purification. Furthermore, the majority of the polyhedra appeared to be intact and only a small number of "broken" organelles were observed. In this regard, we point out that pre-fixation prior to negative staining (as described in the Materials and Methods section) was required to prevent breakdown of the polyhedral organelles during electron microscopy.

SDS-PAGE Analysis

The progress of the polyhedral organelle purification was also followed by SDS-PAGE (Figure 4-2). Purified organelles were found to be composed of at least 14 proteins with molecular masses ranging from 10.6 to 58.6 kDa (Figure 4-2, lane 7). Among these proteins the 10.6, 11.1, 17.6, 27.2, 29.5, 38.5, 42.6, 51.9, and 58.6 kDa proteins appeared to be the most abundant. These major protein components of the polyhedra were not easily observed in the crude lysate (Figure 4-2, lane 2), the B-PER II extracted lysate (Figure 4-2, lane 3), or the 12,000 x g supernatant (Figure 4-2, lane 4). However, they became apparent in the 48,000 x g pellet (Figure 4-2, lane 5) and were further purified following low speed (Figure 4-2, lane 6) and sucrose gradient centrifugation (Figure 4-2, lane 7).

Diol Dehydratase Activity of the Polyhedral Organelles

Since diol dehydratase was previously shown to be associated with the polyhedral organelles (Bobik et al. 1999, Havemann et al. 2002), the activity of this enzyme was also used to monitor the organelle purification (Table 4-1). The specific activity of diol

dehydratase was unchanged by the detergent treatment, but this step was necessary to remove lipid vesicles. The 12,000 \times g centrifugation that followed the detergent treatment resulted in a decrease in the specific activity of diol dehydratase.

Table 4-1. Diol dehydratase activity during organelle purification

Sample	Protein (mg)	Activity (U) ^a	Specific activity (U/mg)	Yield (%)	Fold - purification
Crude extract	500.0	1,346	2.7	100.0	1.0
Detergent/salts treatment	370.6	1,000	2.7	74.3	1.0
12,000 \times g super	357.0	573	1.6	42.6	0.6
48,000 \times g pellet	9.4	194	20.8	14.4	7.7
12,000 \times g super	7.0	195	27.9	14.5	10.3
Sucrose density gradient	0.6	16	27.5	1.2	10.2

^aOne unit activity is defined as the amount of enzyme that catalyzes the formation of 1 μ mol of propionaldehyde per min per mg protein.

However, this step was needed to remove aggregates, which included many organelles that were not released from vesicles by the detergent treatment. Following aggregate removal, the polyhedral organelles were pelleted by centrifugation at 48,000 \times g, resuspended in buffer, and clarified by a second centrifugation at 12,000 \times g. These two steps proved very effective and increased the specific activity of diol dehydratase from 1.6 to 27.9 μ mol/min/mg. The final sucrose density gradient step typically resulted in a slight drop in the specific activity of diol dehydratase (Table 4-1). The reason is uncertain. It may have been due to removal of an unknown factor that enhances diol dehydratase activity or due to partial enzyme inactivation.

For the complete purification, the specific activity of diol dehydratase increased approximately 10-fold, from 2.7 to 27.5 μ mol/min/mg protein (Table 4-1). This indicates

that the polyhedral organelles constitute about 10% of the total cell protein, which is consistent with previous electron microscopy, which showed that these structures occupy a significant portion of the cytoplasm of *S. enterica* grown under the conditions used for organelle purification (Bobik et al. 1999, Havemann et al. 2002).

Other Activities of the Polyhedral Organelles

In addition to measuring diol dehydratase activity of the polyhedral organelles, propionaldehyde dehydrogenase and adenosyltransferase activities were also measured. No adenosyltransferase activity was detected in 50ug of polyhedral organelles however, the specific activity of propionaldehyde dehydrogenase was followed throughout the purification and the results are summarized in [Table 4-2](#). Unlike diol

Table 4-2. Propionaldehyde dehydrogenase activity during organelle purification

Sample	Protein (mg)	Activity (U) ^a	Specific activity (U/mg)	Yield (%)	Fold - purification
Crude extract	515	152	0.295	100	1
Detergent/salts treatment	337.9	205.2	0.607	135	2.1
12,000 x g super	338.8	169	0.50	111	1.7
48,000 x g pellet	5.0	9.6	1.92	6.3	6.5
12,000 x g super	4.3	8.6	2	5.7	6.8
Sucrose density gradient	0.3	1.2	4	0.8	13.6

^aActivities were calculated from the initial rate and one unit activity was defined as the amount of enzyme that catalyzes the formation of 1 µmol of NADH per min per mg.

dehydratase activity, which remained unchanged after detergent treatment, propionaldehyde dehydrogenase activity increased nearly three-fold. By itself, this result would suggest that cell debris and/or lipid membranes may be inhibiting propionaldehyde

dehydrogenase activity by preventing access to the enzyme; however, it is unclear why the activity of propionaldehyde dehydrogenase exhibited an increase without a concomitant increase in diol dehydratase activity. The 12,000 x g spin that followed the detergent treatment resulted in a slight loss of activity which is most likely due to the pelleting of organelles with cell debris. Like diol dehydratase activity, the activity of propionaldehyde dehydrogenase showed a significant increase after high speed centrifugation and clarification (0.50 to 2 U/mg) and a 2-fold increase after separation on a sucrose gradient (2 to 4 U/mg). The approximate 13.6-fold increase of propionaldehyde dehydrogenase activity observed for the complete purification is consistent with idea that polyhedral organelles constitute approximately 10% of the total cell protein.

Western Blot Analysis of Purified Polyhedral Organelles

To examine their composition, western blots were performed on purified polyhedral organelles using antisera against diol dehydratase (PduCDE) and the PduAJOP proteins. An antiserum previously shown to be specific for the PduA protein (Havemann et al. 2002) recognized a single band at 11 kDa ([Figure 4-3, lane 3](#)). This is near the expected value for PduA (9.6 kDa) indicating that this protein is a component of the purified organelles. A second antiserum, shown in a prior study to react with both the PduA and PduJ proteins (Havemann et al. 2002), recognized one band at 11 kDa (PduA) as well as a second band near the predicted molecular mass of PduJ (10.6 kDa) ([Figure 4-3, lane 4](#)). Thus, it appears that the PduJ protein is also an organelle component.

In further serological tests, PduO and PduP antisera were found to recognize proteins of 39 and 52 kDa, as well as some additional proteins. ([Figure 4-3, lanes 6 and](#)

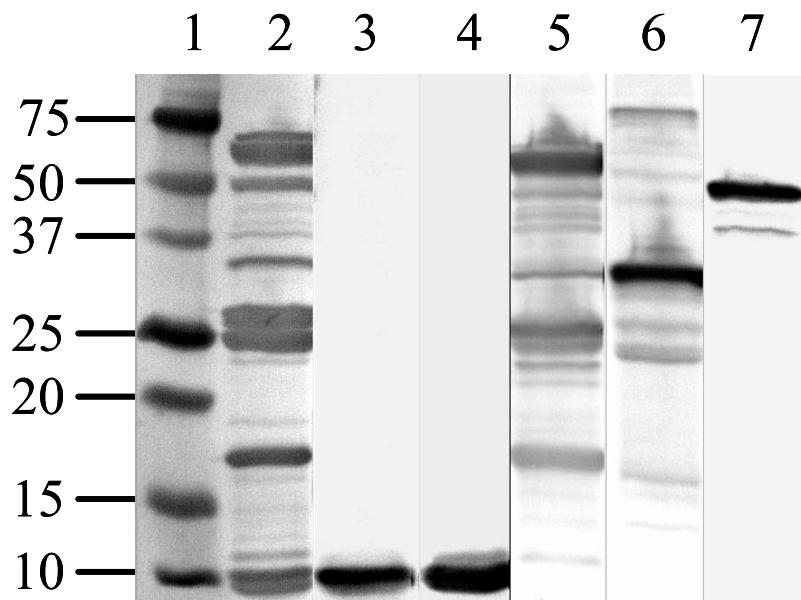


Figure 4-3. Western analysis of purified polyhedral organelles. Lane 1, molecular mass markers; lanes 2-7, each contained 20 µg of purified polyhedral organelles. Lane 2 was stained with Coomassie Brilliant Blue and lanes 3-7 were probed with the following antisera: lane 3, anti-PduA; lane 4, anti-PduAJ; lane 5, anti-diol dehydratase; lane 6, anti-PduO; lane 7, anti-PduP.

7). The proteins at 39 and 52 kDa had molecular masses near the predicted values for PduO and PduP (36.8, and 49 kDa) and were unreactive with pre-immune sera in control experiments indicating that they likely represent the PduO and PduP proteins, respectively. The additional protein bands recognized by the PduO and PduP antisera appeared to be nonspecific since pre-immune sera reacted with protein bands of similar molecular masses (data not shown). Hence, these results indicated that the PduO and PduP proteins are part of the purified organelles.

The reaction of purified organelles with anti-diol dehydratase antibody was also tested. This antibody preparation reacted with proteins of 58.6, 30 and 18 kDa (Figure 4-3, lane 5). These are the approximate sizes of the three subunits of diol dehydratase (60.3, 24.2, and 19.1 kDa). Prior studies showed that the antiserum used reacts with diol dehydratase (Toraya and Fukui 1977, Bobik et al. 1999, Havemann et al. 2002), but clearly there was also some cross-reactivity as a number of other protein bands were recognized. Nonetheless, three protein bands near the expected molecular masses for the three subunits of diol dehydratase were identified supporting the enzyme assays described above which indicated that diol dehydratase is associated with the purified organelles.

Glycoprotein Staining of Organelle Proteins

Prior studies showed that the Csos1 and Csos2 proteins of carboxysomes are glycosylated (Holthuijzen et al. 1986, Baker et al. 1999). Since some components of the *pdu* organelles share homology with the Csos1, we tested the polyhedral organelles purified from *S. enterica* for the presence of glycoproteins. Organelles proteins were separated by SDS-PAGE and stained for glycoproteins using a commercial kit that is

based on the Periodic Acid-Schiff (PAS) staining method (Zacharius et al. 1969). No glycosylated proteins were detected in the polyhedral organelle preparation (20 µg).

Two-Dimensional Electrophoretic Analysis of Purified Polyhedral Organelles

Samples of purified polyhedral organelles were separated by 2D-IEF-SDS-PAGE. Seventeen major (1-12, 16, 18, and 20-22) and five minor protein spots (13-15, 17 and 19) were observed ([Figure 4-5](#)). Each protein present in [Figure 4-5](#) was reproducibly observed following 2D-electrophoresis of three different organelle preparations.

N-Terminal Sequencing

Subsequent to 2D electrophoresis, the identities of the most prevalent protein spots were assigned by Edman sequential N-terminal degradation followed by sequence similarity searching against the *S. enterica* genome ([Table 4-3](#)) and/or peptide mass fingerprinting via MALDI-TOF MS (Table 4-4) with subsequent searching of the Genpept, SwissProt, and NCBI-nr databases using MS-Fit and the NCBI-nr database using ProFound. N-terminal sequencing was carried out on the protein spots 1-8 ([Figure 4-4](#)). The results are summarized in [Table 4-3](#). Spots 1, 6, 7, and 8 were identified as the PduC, PduE, PduA and PduJ proteins, respectively, confirming the results of the western blotting experiments described above. The N-terminal sequence of protein spots 2 and 5 identified these proteins as PduG and PduB, respectively. Surprisingly, spots 4 and 5 corresponded to the PduB protein and a shorter version of this same protein (PduB') that lacked 37 N-terminal amino acids. Examination of the DNA sequence of the *pduB* gene revealed potential start sites for each protein with an appropriate ATG and Shine-Dalgarno sequence; thus it appears that *pduB* and *pduB'* represent overlapping genes although at this time post-translational proteolytic processing remains a possibility. In addition, both of these proteins appear to have the methionine cleaved after translation.

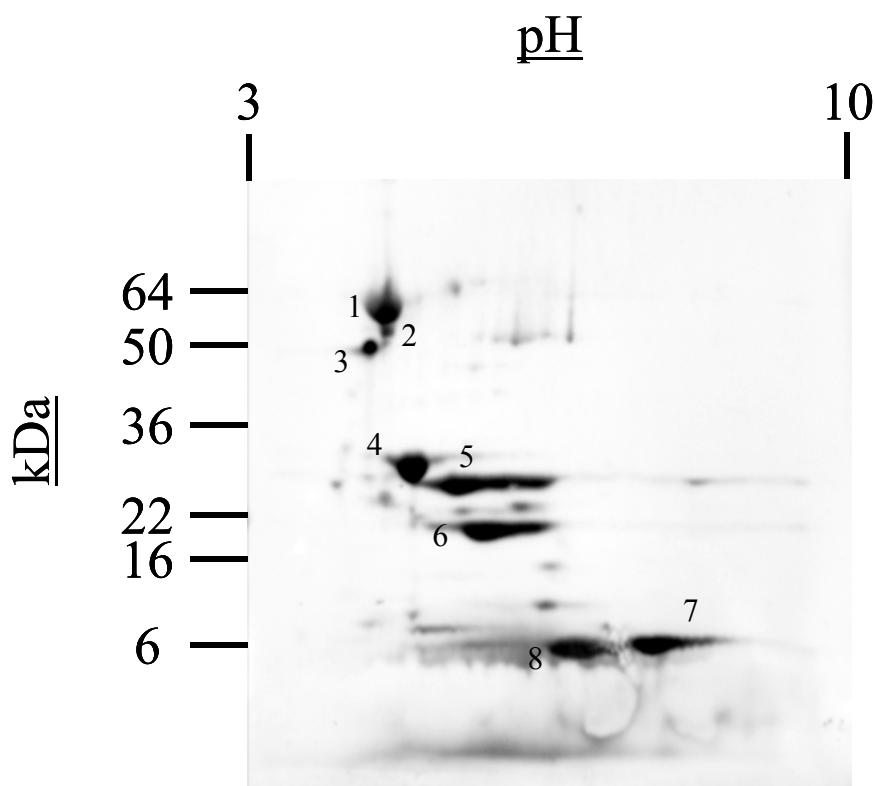


Figure 4-4. Two-dimensional electrophoresis of purified polyhedral organelles of *S. enterica* used for N-terminal sequencing. A purified polyhedral organelle preparation (230 µg of protein) was subjected to 2D-IEF-SDS-PAGE, blotted onto an Immobilon P membrane and then stained with Sypro Ruby. Subsequent to photographing the membrane was stained with Coomassie Blue R-250 and the protein spots were excised and analyzed by N-terminal sequencing as described in the Materials and Methods section. Molecular masses in kilodaltons and the IEF pH is indicated at the left and top of the gel, respectively.

The poor resolution of spots 1, 2 and 3 caused some problems. Spot 2 (PduG) was located below spot 1 (PduC) which seemed incorrect since PduG is has an apparent molecular mass 4 kDa greater than that of PduC. In addition the N-terminal sequence of spot 3 did not match any proteins encoded by the *S. enterica* genome. To resolve these problems the polyhedral organelles were separated on a larger gel and the resulting protein spots were then subjected to MALDI-TOF analyses (see below).

Table 4-3. Organelle proteins identified using N-terminal sequencing

Spot	Experimental N-terminal sequence	Predicted N-terminal sequence*	Corresponding Protein
1	MRSKRFE	MRSKRFE	PduC
2	MRYIAGL	MRYIAGL	PduG
3	AEKELVL	ND	?
4	SSNELVE	MSSNELV	PduB
5	AEKSCSL	MAEKSCS	PduB'
6	MNTDAIE	MNTDAIE	PduE
7	MQQEALG	MQQEALG	PduA
8	MNLALGL	MNNALGL	PduJ

* Prediction based on DNA sequence. ND not determined.

MALDI-TOF Analysis

Proteins corresponding to spots 1-9, 11, 13-15 and 17-20, (Figure 4-5) were excised, digested with trypsin, and analyzed by MALDI-TOF MS and protein mass fingerprinting (Table 4-4). A sample spectrum of spot 11 is shown in Figure 4-6. Both the MS-Fit and Profound Web-based programs were used to analyze the MALDI-TOF spectra. The result of a database search was considered significant if it was ranked as best hit and there was a sequence coverage of at least 25% in either program. Additionally, a MOWSE score (MS-Fit) of at least 1e+003 or a Z-score (Profound) greater than or equal to 1.65 was needed (Baker and Clauser, Zhang and Chait 2000). Based on these analyses, the observed protein spots were identified as the following *S.*

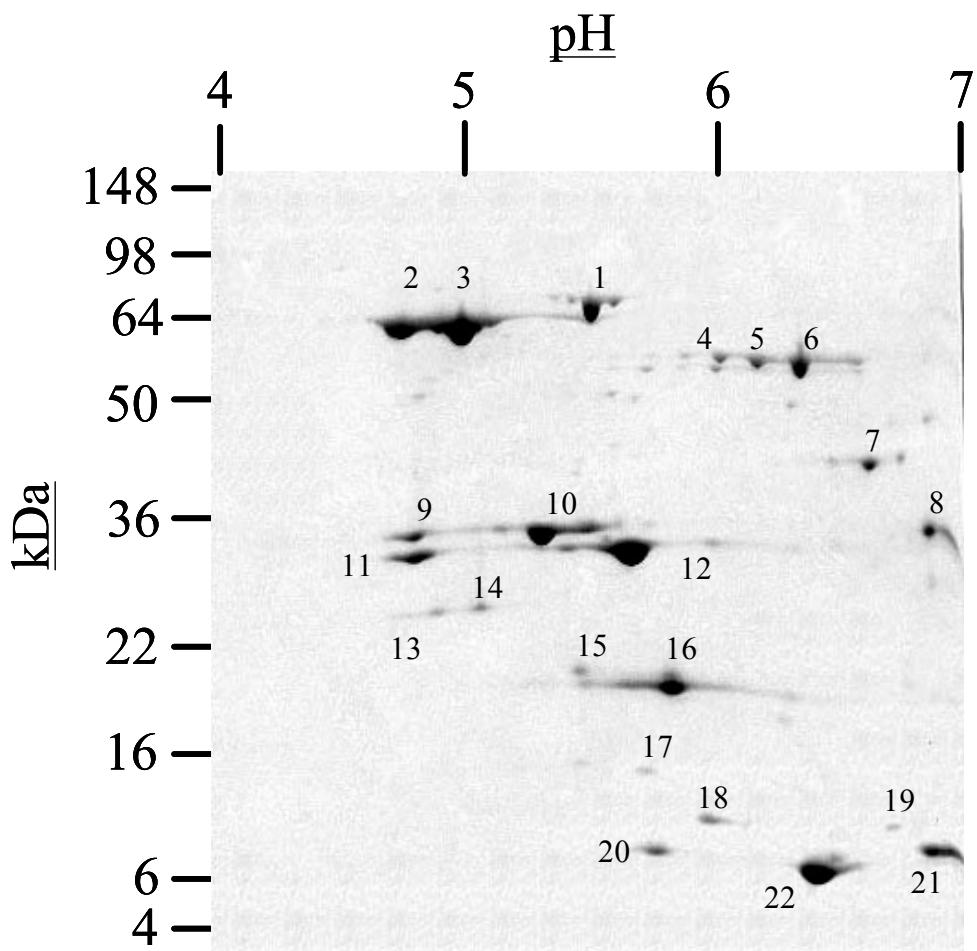


Figure 4-5. Two-dimensional electrophoresis of purified polyhedral organelles of *S. enterica* used for MALDI-TOF analysis. A purified polyhedral organelle preparation (115 µg of protein) was subjected to 2D-IEF-SDS-PAGE and followed by colloidal blue staining. Protein spots were then excised, digested with trypsin and analyzed using MALDI-TOF MS as described in the Materials and Methods section. Molecular masses in kilodaltons and the IEF pH is indicated at the left and top of the gel, respectively.

Table 4-4. Organelle proteins identified by peptide mass fingerprinting

Spot	Identified Protein	NCBI Accession#	MS-Fit			Profound	
			Peptides matched	MOWSE score	% seq. coverage	Z-score	% seq. coverage
1	PduG	16765373	15	5.657E+07	29	2.33	40
2	Pduc	2587029	17	4.674E+07	31	2.43	31
3	Pduc	16765370	6	1.951E+03	16	2.43	52
4	Pdud	16765381	6	8.91E+02	15	1.82	16
5	Pdud	16765381	15	1.622E+05	39	2.33	47
6	Pdud	16765381	15	1.3E+07	36	2.27	54
7	Pdud	16765380	10	3.331E+06	43	2.35	52
8	Pdud	16765371	7	1.371E+04	28	2.43	22
9	Pdud	2587033	26	3.567E+04	47	2.43	44
10	ND	-	-	-	-	-	-
11	Pdud'	16765369	8	3.43E+03	43	2.03	44
12	ND	-	-	-	-	-	-
13	Pdud	16765376	6	2.42E+03	53	1.74	39
14	Pdud	16765376	6	2.42E+03	53	2.12	39
15	Pdud	16765384	9	2.209E+05	42	2.43	39
16	ND	-	-	-	-	-	-
17	U	-	-	-	-	-	-
18	Pdud	16760987-	16	1.262E+08	63	1.74	63
19	Pdud	16765374	4	*	40	2.43	28
20	Pdud	16760978	8	2.24E+05	78	2.33	78
21	ND	-	-	-	-	-	-
22	ND	-	-	-	-	-	-

The spot column refers to the numbers used to mark the protein in [Figure 4-5](#). Both the MS-Fit and Profound programs were used to analyze the MALDI-TOF MS data. A database search result was considered significant if it had a MOWSE score of at least 1e+003 (MS-Fit) or a Z score of at least 1.65 (Profound) and a sequence coverage of at least 25%. The peptides matched was provided by MS-Fit. The NCBI database accession numbers were determined using Profound. The percent sequence coverage determined using each of these programs is also displayed. ND, not determined; U, unsatisfactory; *, not found, the peptides matched and %sequence coverage was based on a digest the Pdud protein using MS-Digest.

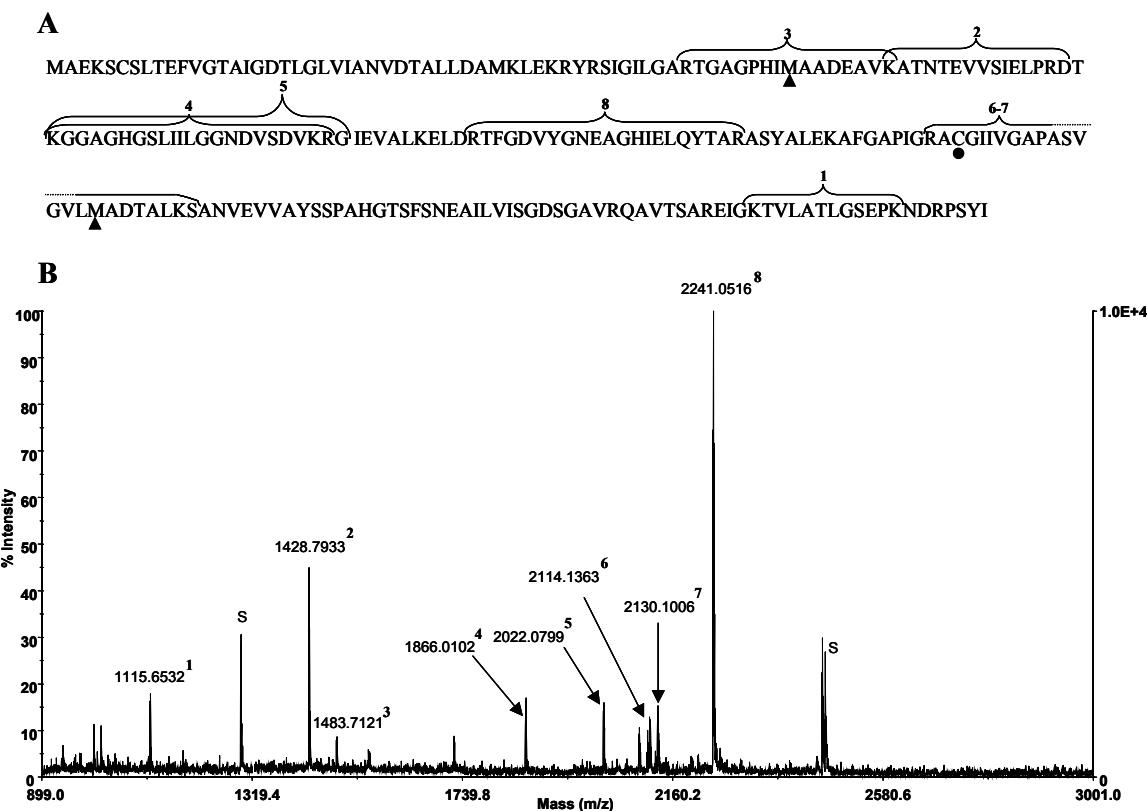


Figure 4-6. MALDI-TOF MS peptide map of spot 11. A. Amino acid sequence of PduB. Eight peptides identified by peptide mass fingerprinting are indicated in the protein sequence. Arrows indicate oxidized methionine residues and the dot indicates a cysteine residue which has been carbamidomethylated. Fragments 6 and 7 indicate same peptide fragment however fragment 7 contains an oxidized methionine which accounts for the mass difference. B. The MALDI-TOF MS spectrum of $[M + H^+]$ molecular ions from the peptides generated by tryptic digestion. S indicates a standard used to calibrate the instrument.

enterica proteins: PduC (2, 3); PduP (5, 6); PduD (8); PduB (9, 10); PduB' (11,12); PduK (13, 14); PduT (15); PduU (18); PduH, and PduJ (20). Although the sequence coverages for spot 4 provided by MS-Fit and Profound were 15 and 16% respectively, the comparison of its spectra with those of spots 5 and 6 indicated that all three of these spots are the PduP protein. The low sequence coverage may be attributed to the quantity of protein used for analysis since spot 4 was visibly less in amount than either spots 5 or 6 and only 6 peptides were matched compared to 15 in spots 5 and 6. Some disparities were noted between the results of the two mass fingerprinting programs and these differences are most likely a result of the different databases searched by MS-Fit or differences in the programs themselves. Using MS-Fit, only searches conducted with the spectra for spots 7, 13, and 14 returned significant matches for Pdu proteins found in the NCBI-nr database. Matches for the remainder of the proteins were found primarily using the Genpept database except for spots 9,11, and 18, which had significant matches in the Swiss-Prot database. Interestingly, Profound was able to find a significant match for each of the Pdu proteins using only the NCBI-nr database. Also of note is the finding that PduH was not identified using MS-Fit, however a theoretical digest of the protein using MS-digest (<http://prospector.ucsf.edu>) and a manual comparison of the resulting peptide fragments and the spectra resulted in a match of 4 peptides and a sequence coverage of 40%. In several instances more than one protein spot corresponded to the same *pdu* protein indicating that some organelle proteins existed in different forms during electrophoresis. This could have resulted from incomplete denaturation of the polyhedral organelles prior to isoelectric focusing, from the tight association of lipids or proteins, or from covalent modification. To break down possible non-covalent interactions, dodecyl-

maltoside was included in the isoelectric focusing step and the 2-D electrophoresis was repeated. Under these conditions single spots were observed for PduC, PduB, and PduB', but PduK, PduJ and PduP were still represented by multiple spots (data not shown). Hence, in the case of PduK, PduJ and PduP, post-translational modification is a possibility.

Densitometry, Molecular Mass, and pI Values of Polyhedral Organelle Proteins Separated by Two-Dimensional Electrophoresis

Following 2D-electrophoresis of the purified organelles, the molecular mass, and pI values for each organelle protein were determined. In addition, protein spots were quantitated by densitometry so that the relative abundance of each organelle protein could be determined ([Table 4-5](#)).

The pI values of organelle proteins were determined from the gel pictured in [Figure 4-5](#), which utilized a linear gradient (pH 4-7). These values were then compared to pI values calculated from sequence analyses based on the protein identifications described above. In general, the observed and calculated pI values were similar. However, the pI values of PduA and PduD could not be accurately determined experimentally because both proteins focused at the upper limit of the pH range for the gel employed (pH 7) and both have calculated pI values above 7. Also spot 4 was not observed in the previous gels thus its identity and pI were not determined.

Densitometry and molecular weight determination was performed on a 2D gel that included dodecyl-maltoside in the first-dimension (to reduce protein streaking) and that had been stained Sypro Ruby which has an extended linear range ([Figure 4-7](#)) (Berggren et al. 2000). Based on mass the PduA shell protein and the putative structural proteins PduBB'J by mass make up the bulk of the polyhedral organelle while PduKTU are minor

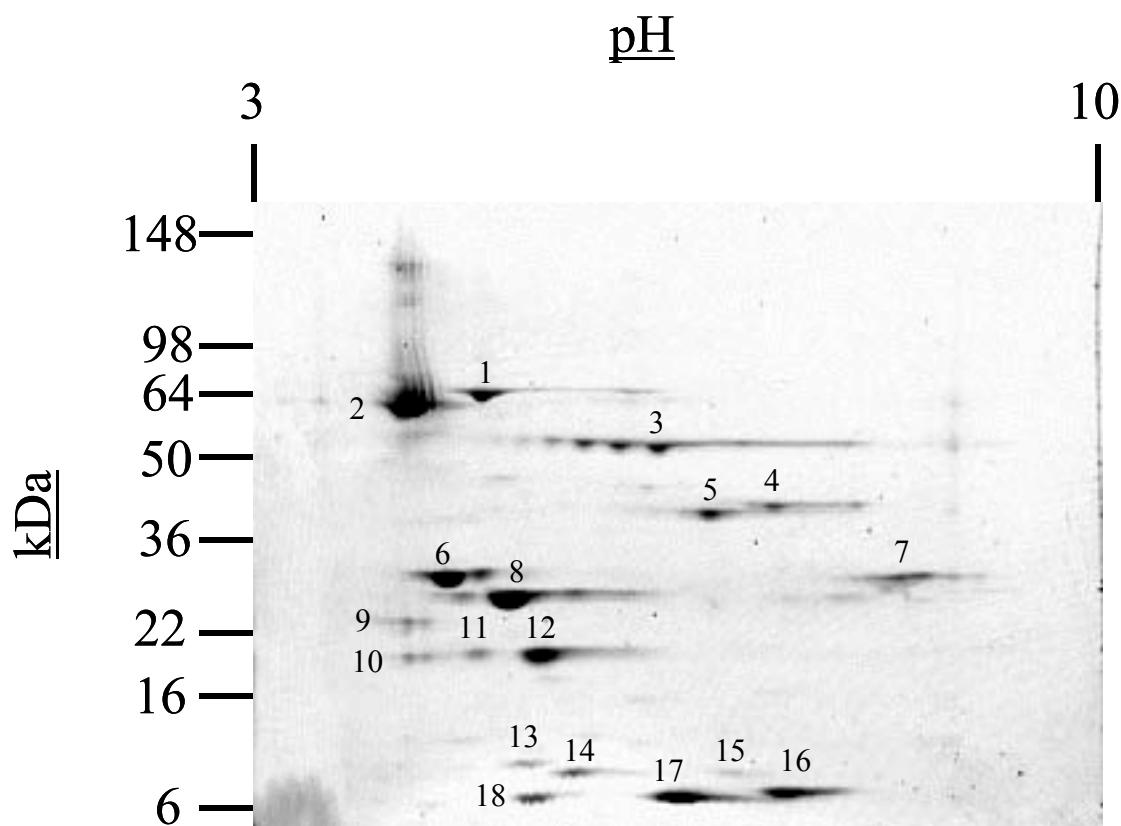


Figure 4-7. Densitometry of the polyhedral organelle proteins. A purified polyhedral organelle preparation (17 µg of protein) was subjected to 2D-IEF-SDS-PAGE and followed by Sypro Ruby staining. The inclusion of dodecyl maltoside in the IEF buffer helped resolve streaking seen in protein spots 2, 6, and 8. The numbers correspond to the protein spots in [Table 4-5](#). Molecular masses in kilodaltons and the IEF pH is indicated at the left and top of the gel, respectively.

Table 4-5. Molecular mass, pI, and densitometry analyses

Protein	Observed Molecular mass (kDa)	Calculated Molecular mass (kDa) ^a	Observed pI	Calculated pI	%Total Protein	Molar Ratio
Pdu G	68.8	64.5	5.5	5.5	5.4	2
Pdu C	65.3	60.3	4.8-5*	5	20.8	8.5
Pdu P	52.9	49.0	6.0-6.4*	6.3	8	4
Pdu O	36.3	36.8	6.6	7.3	3.6	2.5
PduB	25.6	28.0	4.8-5.3*	5.2	12.8	11.5
Pdu D	25.6	24.2	6.9	8.9	4.8	5
Pdu B'	23.0	24.0	4.8-5.7*	5.6	12.1	12.5
Pdu K	16.4	16.8	4.9-5.3*	5.2	1.6	2.5
Pdu T	16.4	19.1	5.5	5.9	1.6	2
Pdu E	16.4	19.1	5.8	5.7	7.9	10.3
Spot 17	9.0	ND	5.7	ND	0.8	ND
Pdu U	8.6	12.5	6	6.1	1.5	3
PduH	8.6	13.4	6.8	7	0.6	1
Pdu A	7.7	9.6	6.9 ^c	7.4	7.5	19.5
Pdu J	7.5-7.7	9.0	5.8-6.4*	6.5	11	30.5

Polyhedral organelles were separated by isoelectric focusing and then by SDS-PAGE. The isoelectric point of each spot was calculated from the gel pictured in [Figure 4-5](#). Both molecular masses and relative protein amounts were calculated from a similar gel which contained the detergent dodecyl-maltoside resulting in greatly reduced streaking exhibited by some protein spots 2, 3 and 9-12 ([Figure 4-7](#)). *Where multiple spots were found to represent the same protein a range of pI values was used.

components. The molar ratios also suggest that PduABB'J are major constituents of the polyhedral organelles (J:A:B':B= 15:10:7:6).

The approximate molar ratios for the subunits of diol dehydratase (PduCDE) and the putative diol dehydratase reactivating factor (PduGH) were 2:1:2 and 2:1, respectively. These ratios are different than the known subunit compositions of diol dehydratase (2:2:2) (McGee et al. 1982), and that proposed for an analogous reactivating factor from *Klebsiella oxytoca* (2:2) (Toraya and Mori 1999). This disparity is most likely due to dye-binding variability, however the possibility that the composition of diol

dehydratase and its reactivating factor differ when inside the polyhedral organelles cannot be ruled out.

If the known subunit composition of diol dehydratase were assumed then propionaldehyde dehydrogenase would be present in a 2-fold lower amount than the diol dehydratase suggesting that it is a monomer. In addition the enzymes required for cofactor regeneration, adenosyltransferase and the reactivating factor components, would be present at amounts 4-fold lower than diol dehydratase. Together these data suggest that the enzymes associated with the polyhedral organelles are present in amounts sufficient for them to function together.

Unidentified Proteins of the Purified Polyhedral Organelles

Each protein spot in [Figure 4-5](#) was assigned an identity, with the exception of 19. This protein spot had an apparent pI of 6.8 and an observed molecular mass of 8.6 kDa. These values did not match well with any known *pdu* protein and protein mass fingerprinting did not match this spot to any protein found in the *S. enterica* genome. The 37.9 kDa protein corresponding to spot 4 in [Figure 4-7](#) also was analyzed since it was not observed in previous preparations used to identify protein by N-terminal or MALDI-TOF analysis. This may have been due to poor transfer of spot 4 to the membrane used for N-terminal sequencing ([Figure 4-4](#)) and poor staining by colloidal blue in the gel used for MALDI-TOF analysis ([Figure 4-5](#)). Although the pI of spot 4 was not determined, its position to the right of PduO indicates that it has a pI higher than that of the PduO protein (pI=7.3). Based on this the only possible Pdu protein candidate for the spot 4 is PduX (pI=7.8); however the molecular mass of PduX is 5kDa smaller than that of spot 17 (37.9 kDa). Thus, either PduX runs aberrantly during SDS-PAGE or spot 4 is not encoded by the *pdu* operon.

Discussion

Previously, we established that *S. enterica* forms polyhedral organelles during Ado-B₁₂-dependent growth on PD (Bobik et al. 1999, Havemann et al. 2002).

Immunolabeling studies demonstrated that these organelles consisted of a protein shell (partly composed of the PduA protein), the majority of the cell's B₁₂-dependent diol dehydratase, and additional unidentified proteins (Bobik et al. 1999, Havemann et al. 2002). In this report, we purified the polyhedral organelles involved in PD degradation by *S. enterica* and showed that they consist of at least 16 proteins ([Table 4-5](#)).

The best studied polyhedral organelles, the carboxysomes of *Halothiobacillus neapolitanus*, are composed of at least 9 proteins: six shell proteins, Csos1A, Csos1B, Csos1C, Csos2A, Csos2B and Csos3; one protein of unknown function; and the large and small subunits of RuBisCO, CbbL and CbbS (Cannon and Shively 1983, Holthuijzen et al. 1986). Thus far, the only protein shown to be part of the shell of the *S. enterica* organelles is the PduA protein (Havemann et al. 2002), which is a homologue of the Csos1A, Csos1B, and Csos1C proteins (Cannon and Shively 1983, Holthuijzen et al. 1986, Zhu and Lin 1989). However, the PduJKT proteins also have at least 27% amino acid sequence identity to Csos1 (Bobik et al. 1999), and all are constituents of the *S. enterica* organelles (this study) suggesting that these proteins may also be shell components of the *pdu* organelles. Likewise, the PduB, PduB' and PduU organelle proteins (this study) are distantly related to Csos1 (Cannon et al. 2001) and hence may also serve as shell components. If so, based on mass PduB' and PduB would be the first and second most abundant structural components of the *S. enterica* organelles, respectively.

In addition to the putative shell proteins, four enzymes were shown to be major components of the purified *pdu* organelles: Ado-B₁₂-dependent diol dehydratase (PduCDE), propionaldehyde dehydrogenase (PduP), adenosyltransferase (PduO), and a putative diol dehydratase reactivating factor (PduGH). As a group, these enzymes are sufficient to mediate the conversion of PD to propionyl-CoA. Ado-B₁₂-dependent diol dehydratase catalyzes the conversion of PD to propionaldehyde, which is then converted to propionyl-CoA by the CoA- dependent propionaldehyde dehydrogenase (Abeles and Lee Jr. 1961, Toraya et al. 1979, Obradors et al. 1988). Adenosyltransferase and the PduGH reactivating factor are needed to maintain diol dehydratase in an active form (Johnson et al. 2001). Thus, the *pdu* organelles include all the enzymatic activities needed for the conversion of PD to propionyl-CoA.

The finding that the *pdu* organelles include diol dehydratase (aldehyde producing) and propionaldehyde dehydrogenase (aldehyde consuming) is consistent with the prior proposal that these organelles function to minimize aldehyde toxicity (Havemann et al. 2002). In the model represented by [Figure 4-8](#), PD crosses the organelle shell and is transformed to propionaldehyde by the PduCDE diol dehydratase. The propionaldehyde is then sequestered within the organelle until it is converted to propionyl-CoA by the PduP aldehyde dehydrogenase before it diffuses out of the organelle. Since aldehydes are cytotoxic, sequestering of propionaldehyde might serve to protect sensitive cytoplasmic components. However, currently, it is unclear how the shell of the organelle would retain propionaldehyde while allowing higher molecular weight compounds such as the required cofactors for the organelle enzymes [Ado-B₁₂, CoA, and NAD(H)] to pass. Alternatively, the inclusion of diol dehydratase and propionaldehyde dehydrogenase in

the *pdu* organelles 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* organelles minimize aldehyde toxicity by limiting aldehyde production through control of Ado-B₁₂ availability (Havemann et al. 2002). Such a mechanism would be improved by either aldehyde channeling or sequestration, and, clearly, the *pdu* organelles are sufficiently complex that multiple mechanisms could be at work to fine tune aldehyde production and consumption.

Further clues as to the structure, function and assembly of the *S. enterica* organelles might lie in the fact that our analyses of the purified organelles did not detect the PduLMNQSVWX_n proteins. These proteins are encoded by the *pdu* locus suggesting that they function in PD degradation, but they are either absent from the purified *pdu* organelles or present at levels below detection. Based on sequence similarity, the PduQ and PduW proteins are thought to be a propanol dehydrogenase and a propionate kinase involved in the PD degradative pathway ([Figure 4-8](#)). Apparently, these steps of PD degradation occur outside the organelles. The PduN protein has homology to the proteins encoded by *orfA* and *orfB* of *H. neapolitanus*, the *Synechocystis* CcmL protein, and other related proteins that are required for carboxysome formation (Bobik et al. 1999, Cannon et al. 2002). Like PduN, the products of *orfA* and *orfB* of *H. neapolitanus* were not detected in purified preparations of organelles. Furthermore, mutations in the *ccmL* gene result in high-CO₂- requiring mutants that synthesize elongated carboxysomes (Price and Badger 1989, Price and Badger 1991, Price et al. 1993). Together these results suggest that PduN and homologues are either only transiently involved in organelle assembly or

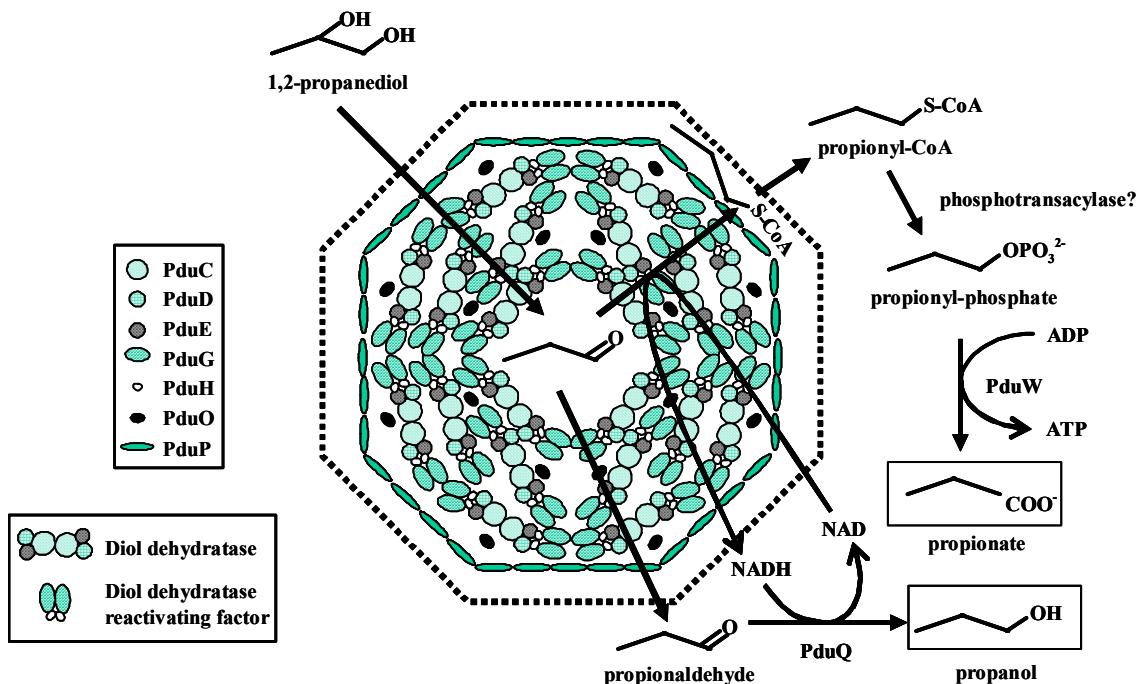


Figure 4-8. A model of the *pdu* organelle. The dashed line indicates the shell of the organelle which is composed of the PduA protein and perhaps the PduBB'JKTU as well. The enzymes of the organelle are graphically represented and are indicated in the legend. However, their placement in the organelle has not been determined experimentally. The pathway of PD degradation is also shown. It is proposed that PD enters the organelle where it is converted to propionaldehyde by the PduCDE diol dehydratase. The propionaldehyde is channeled to the PduP aldehyde dehydrogenase and converted to propionyl-CoA which diffuses out of the organelle and is converted to propionate with the production of ATP. Part of the aldehyde is oxidized to propionyl-CoA as it diffuses out of the organelle and part is reduced to propanol outside of the organelle. Further conversion of propionyl-CoA occurs outside of the organelle. Propionaldehyde which escapes channeling is sequestered within the organelle to protect cytoplasmic cellular components. However, some propionaldehyde escapes into the cytoplasm where it is converted into 1-propanol. The putative alcohol dehydrogenase (PduQ), propionate kinase (PduW), and phosphotransacylase, were not detected in the organelles purified for this study, and here are shown as part of the PD degradation pathway that occurs outside of the polyhedra.

only minor components of their respective polyhedral organelles. Five additional *pdu* proteins that were undetectable in purified organelles (PduLMSVX) are of unknown function and lack convincing homology to proteins of known function currently in the NCBI nr database. These proteins might be needed for organelle assembly, but are absent from mature structures. Alternatively, they might encode additional enzymes needed for PD degradation or B₁₂ metabolism that function outside the polyhedral organelles.

Overall, the analyses presented in this report indicate the *pdu* organelles consist of a shell composed of 4-7 proteins that encase 4 enzymes. The shell of the *pdu* organelles appears to be related to the shell of carboxysomes since 4 *pdu* proteins have homology to the carboxysome shell protein Csos1A (Shively et al. 1998, Bobik et al. 1999). However, the functions of carboxysomes and the *pdu* organelles, at present, appear to be unrelated. Carboxysomes are involved in autotrophic CO₂ fixation whereas the *pdu* organelles apparently mediate the conversion of PD to propionyl-CoA. The fact that two distinct metabolic process have been found to occur within polyhedral organelles raises two interesting questions: how many different metabolic processes occur within protein-bound organelles, and how widely distributed are such organelles in nature. Recent electron microscopy and bioinformatic studies indicate that four different metabolic processes occur within protein-bound organelles and that such structures are relatively widespread in nature. Carboxysomes are found in a number of photo- and chemoautotrophs (Shively et al. 1998, Cannon et al. 2001, Cannon et al. 2003). Electron microscopy studies have shown that *Salmonella*, *Klebsiella*, and *Citrobacter* form polyhedral organelles during growth on PD (Shively et al. 1998, Bobik et al. 1999,

Havemann et al. 2002)(H.C. Aldrich, T.A. Bobik, D.S. Williams, R.J. Busch, Abstr. Annu. Meet. Am. Soc. Microbiol. 1998, abstr. 140J-2); and the analyses of sequenced genomes indicate that *pdu* organelles are also formed by *Listeria*, *Lactobacillus*, and *Clostridium*. A related but uncharacterized organelle involved in Ado-B₁₂-dependent ethanolamine utilization (*eut*) has been observed in *Salmonella*, *Escherichia*, and *Klebsiella* grown on ethanolamine (Shively et al. 1998)(H.C. Aldrich, T.A. Bobik, D.S. Williams, R.J. Busch, Abstr. Annu. Meet. Am. Soc. Microbiol. 1998, abstr. 140J-2), and genomic analyses indicate that *Fusobacterium*, *Listeria*, and *Clostridium* also produce *eut* organelles. In addition, based on bioinformatic studies it appears that *Desulfitobacterium hafniense* may also possibly form polyhedral organelles. The genome of this organism includes multiple homologues of organelle shell genes interspersed with genes of unknown function suggesting that yet a fourth metabolic process that occurs within a polyhedral organelle.

Importantly, the possibility exists that a larger number of metabolic processes might occur with protein-bound organelles. Such organelles might have escaped prior notice because their observation would require electron microscopy of cells grown under specialized conditions as is the case for both *pdu* and *eut* organelles. Furthermore, bioinformatic analyses would fail to identify protein-bound organelles that lack homologues of known organelle proteins. Thus, protein-bound organelles are involved in 3-4 or perhaps more metabolic processes.

CHAPTER 5 CONCLUSIONS

The discovery of homologues to carboxysome proteins in the *eut* and *pdu* operons and the subsequent observation of polyhedral organelles in cells grown under conditions which induce these operons, sparked interest in determining the role of these organelles in B₁₂-dependent metabolism. Prior to the work conducted in this dissertation little was known about the polyhedral organelles except that they were produced in cells grown on PD. This dissertation focused on elucidating the structure and function of the polyhedral organelles involved in the AdoB₁₂-dependent degradation of PD.

Conditions Required for the Formation of the Polyhedral Organelles

The first part of this study (Chapter 2) investigated the conditions required and identified some of the genes involved in the formation of the polyhedral organelles in *S. enterica*. Previous studies on the regulation of the *pdu* operon demonstrated that the *pdu* genes are induced aerobically during growth on poor carbon sources but are maximally expressed during anaerobic growth on a poor carbon source in the presence of PD (Bobik et al. 1992, Chen et al. 1995). Studies described here show that polyhedral organelles are formed under similar growth conditions.

To examine the role of polyhedral organelles in the degradation of PD, a time course of organelle formation was performed. Using electron microscopy, polyhedral organelles were observed during growth of *S. enterica* on PD and were found to appear just prior to entrance into the log phase of growth. The number of polyhedral organelles appeared highest during mid-log phase, and even though the numbers dwindled in the late

logarithmic phase, polyhedral organelles were observed even in dead cells. These findings indicate that the presence of the polyhedral organelles is important to degradation of PD.

Catabolism of PD was not required for the formation of the polyhedral organelles, and cells grown either aerobically or anaerobically on a poor carbon source, but not on a rich carbon source formed organelles. It was also found that anaerobiosis was not required for the formation of the polyhedral organelles. Interestingly, cells grown on glycerol under anaerobic conditions, a condition previously shown to highly induce expression of the *pdu* operon (Bobik et al. 1992, Chen et al. 1995), produced very few organelles suggesting that PD may play some role in their formation in addition to gene induction.

Genes Involved in the Formation of the Polyhedral Organelles

The role of the *pduABCDE* genes in organelle formation was also examined. From a pool of mutants, five strains containing insertion mutations in either the *pduA*, *pduB*, or the *pduC* genes were selected and examined for the production of polyhedral organelles during growth on either PD or PD/succinate minimal medium. From these studies it was evident that the *pduA* and *pduB* genes were essential for the formation of the polyhedral organelles, while studies conducted with the *pduC* gene, which encodes the large subunit of diol dehydratase, gave mixed results that merited further study.

To further study the role of diol dehydratase in organelle formation, it was decided that a precise non-polar deletion of the *pduCDE* genes should be constructed. These genes encode the large, medium, and small subunits of diol dehydratase. The deletion mutant was examined for the formation of polyhedral organelles and results indicated that expression of diol dehydratase was not required for the formation of the polyhedral

organelles. Thus, the formation of the polyhedral organelles does not appear to be dependent upon their association with the diol dehydratase enzyme and consequently does not appear to require the *pduC* gene.

Complementation studies conducted in this strain demonstrated that plasmid encoded diol dehydratase could be packaged into polyhedral organelles. However, complementation studies conducted in *pduA* and *pduB* insertion mutants were unsuccessful. Aberrant structures were observed in both instances, which could be interpreted to suggest that the downstream expression from the tetracycline-inducible promoters of the TPOP insertion was not similar enough to wild type levels to support formation of the polyhedral organelles. Alternatively, the proteins involved in polyhedral organelle formation could be required in strict ratios for correct assembly or the expression of downstream genes could be dependent upon expression of the *pduA* and *pduB* genes. The answers to these questions could be answered better by conducting complementation studies in precise nonpolar deletion mutants.

PduA is a Shell Protein Required for the Formation of the Polyhedral Organelles

The second part of this study (Chapter 3) investigated the role of the PduA protein in the formation of the polyhedral organelle. Experiments conducted using antiserum generated against purified recombinant PduA protein demonstrated that the PduA protein localized to the periphery of the polyhedral organelles, indicating that it was a component of the organelle shell. Taking cues from the studies conducted in Chapter 2, complementation of the *pduA* gene was studied in a precise nonpolar deletion mutant. Expression of plasmid-encoded PduA in this strain resulted in the formation of both normal and aberrant polyhedral organelles. Although not all of the polyhedral organelles were perfectly formed, the degree of complementation seen in this deletion strain was

significantly better than that observed in the insertion mutant suggesting that abnormal downstream expression was the culprit in the complementation studies conducted in the *pduA*::TPOP insertion mutant. The occurrence of some aberrant polyhedral organelles was later found to be due to the inadvertent deletion of the start codon for the *pduB* gene in this strain (see below).

The *pduA* deletion provided a nonpolar mutant to study the phenotype of a polyhedral organelle minus strain. Growth studies indicated that an extended period of “interrupted” growth occurred in the mutant and not in the wild type. Physiological studies conducted on the *pduA* mutant indicated that this lag was related to the amount of PD included in the growth medium suggesting that buildup of a toxic intermediate may be the cause of the “interrupted” growth. Additional physiological studies demonstrated that the *pduA* mutant grew at faster initial rates than the wild type when supplied with low amounts of B₁₂ suggesting that the polyhedral organelles may act as B₁₂ barriers. This finding suggests that the polyhedral organelles may play a role in limiting aldehyde toxicity by moderating the rate of aldehyde production via control of B₁₂ accessibility.

Purification of the Polyhedral Organelles

Like the carboxysome, study of the polyhedral organelles of *S. enterica* would be greatly facilitated by a purification procedure that provides homogenous, stable organelles. The third part (Chapter 4) of this study dealt with the development of such a purification scheme. As a starting point, the procedure developed by Cannon et al. for the purification of carboxysomes from *Halothiobacillus neapolitanus* was used (Cannon and Shively 1983). Like this procedure, sonication proved to be the best method for breaking cells for polyhedral organelle purification. Subsequent centrifugation however resulted in pelleting of the polyhedral organelles with the cell debris and unbroken cells

when buffers including magnesium were used. Subsequent empirical studies, determined that exclusion of magnesium from the buffer and the inclusion of sodium chloride and the proprietary detergent, B-PER II, were essential for the separation of lipid vesicles from the polyhedral organelles. The reintroduction of magnesium into the subsequent buffers used in these experiments was based solely on analogy to the buffers used in the carboxysome purifications and prior knowledge that magnesium helps stabilize multi-protein complexes. Future refinements of the purification may determine that magnesium is not a requirement for polyhedral organelle stability.

The Polyhedral Organelles are Composed of at Least 14 Proteins

Subsequent to purification of the polyhedral organelles, fourteen constituent proteins were identified: PduCDE (diol dehydratase), PduO (adenosyltransferase), PduP (propionaldehyde dehydrogenase), PduGH (diol dehydratase reactivating factor), and PduABB'JKTU (carboxysome shell protein homologues). The identification of the diol dehydratase, propionaldehyde dehydrogenase, and diol dehydratase reactivating factor is consistent with the idea that the polyhedral organelles are involved in aldehyde production. The absence of the remaining pathway enzymes required for PD degradation was interesting and is consistent with the idea that the polyhedral organelles function to detoxify propionaldehyde.

The finding of a longer PduB protein was surprising since no similar protein could be found in the LT2 genome. After further investigation it was found that a compression in the *pdu* operon sequence published previously by Roth's laboratory ((Chen et al. 1994)) masked the start site for this protein. This offered some explanation for the complementation difficulties encountered with PduA mutants conducted in Chapters 2 and 3. Apparently strain BE182, which was used to conduct complementation studies in

Chapter 2 and was thought to contain a precise nonpolar deletion of the *pduA* gene, in fact appeared to contain a deletion of both the *pduA* gene and the start of the *pduB* gene. Although this finding does not negate the results of the physiological experiments conducted in Chapter 3, it does raise questions as to whether a *pduA* deletion alone would result in a polyhedral organelle minus phenotype.

A Model for the Polyhedral Organelles of *S. enterica*

In Chapter 2, a hypothesis was made which stated that the polyhedral organelles serve to moderate aldehyde production by controlling access to B₁₂. Several criteria were used as the basis of this hypothesis. First, mutants unable to synthesize polyhedral organelles exhibited a period of growth arrest at a time where wild type cells were entering the logarithmic growth phase. Next, the duration of the growth lag was found to be directly related to the amount of PD included in the growth medium. Lastly, polyhedral organelle minus mutants grew at faster initial rates than the wild type at low B₁₂ concentrations suggesting that the polyhedral organelle served as a barrier to B₁₂ entry. Together these findings were interpreted to mean that mutants grew at faster initial rates, especially under conditions of high substrate or coenzyme concentrations, until enough of the unidentified toxic intermediate was made to halt growth. A likely candidate for the toxic intermediate was propionaldehyde, the product of Ado-B₁₂-dependent diol dehydratase and also a known cytotoxin.

With the identification of the major organelle proteins in Chapter 4, a model for the polyhedral organelles was proposed ([Figure 4-8](#)). According to the model, PD enters the polyhedral organelle where it is converted to propionaldehyde, which is then oxidized to propionyl-CoA. The sequestration or channeling of propionaldehyde inside the organelle assures that the majority of this compound (the proposed toxic intermediate) is converted

to propionyl-CoA, thus protecting the cytoplasmic contents. This model is supported by the identification of the diol dehydratase and propionaldehyde dehydrogenase enzymes among the organelle proteins. In addition to these two enzymes, both components of the putative diol dehydratase reactivating factor and an adenosyltransferase were identified; however, no reductase was identified. A scenario where B₁₂ use is limited by the lack of reduced Ado-B₁₂ precursors is therefore a viable possibility for the regulation mechanism of diol dehydratase activity, and consequently, aldehyde production.

In the model, the first two steps of the pathway, the conversion of PD to propionaldehyde and its subsequent oxidation to propionyl-CoA, are proposed to occur within the polyhedral organelle while reduction to propanol and further conversion of propionyl-CoA to propionic acid are thought to occur outside of the organelle. Alternatively, the enzymes required for these latter reactions could have been missed due to low quantities; however, this seems unlikely since it seems reasonable that the pathway enzymes would be present in roughly equal amounts. A third possibility is that these proteins are associated with the outside of the organelle and are lost during purification due to either interruption of ionic bonds or hydrophobic interactions by use of sodium chloride or detergent. Further study will be needed to determine the location of these enzymes. If one assumes that the placement of the enzymes is correct, the PduQ protein may serve to convert aldehyde which has leaked out of the polyhedral organelles into the less toxic metabolite, propanol, which is then subsequently excreted from the cell.

Future Experimentation

The development of a purification procedure for the polyhedral organelles opens the door for many future experiments. Biochemical studies of organelle function and mechanisms are now possible. Specifically, the comparison of activities of enzymes

normally associated with the organelles can now be compared to those of the free enzymes to determine if association of these enzymes with the organelle alters any of their characteristics.

Further elucidation of the polyhedral organelle function is also now possible. A logical starting point would be to examine the enzyme activities of intact organelles *in vitro* and compare these findings with the results of similar experiment using organelles from mutants lacking selected organelle proteins. In addition, since purification was carried out under aerobic conditions and the *pdu* operon has been shown to be maximally induced under anaerobic conditions (Bobik et al. 1992, Chen et al. 1995), the purification and investigation of polyhedral organelles from cells grown under anaerobic conditions could potentially reveal important aspects of the polyhedral organelle function. Furthermore, the purification procedure described in this dissertation will most likely be adaptable for use in purifying polyhedral organelles associated with the degradation of ethanolamine and other yet to be discovered processes with only minor modification.

Another intriguing question that was left unanswered is how enzymes are packaged into these polyhedral organelles. In Chapter 2 it was shown that plasmid-encoded diol dehydratase could be packaged into the polyhedral organelles however the means by which diol dehydratase was either tagged for packaging or recognized by the polyhedral organelle was not examined thus leaving much ground in this area uncovered. Presumably the packaging of RuBisCO and diol dehydratase within a proteinaceous shell alters the characteristics of these enzymes or the processes in which they are involved. Consequently, elucidation of the polyhedral organelle function and the mechanism by

which these enzymes are packaged into these structures could have industrial applications.

LIST OF REFERENCES

- Abeles, R. H., and H. A. Lee Jr.** 1961. An intramolecular oxidation-reduction requiring a cobamide coenzyme. *J. Biol. Chem.* **236**:2347-2350.
- Ailion, M., T. A. Bobik, and J. R. Roth.** 1993. Two global regulatory systems (Crp and Arc) control the cobalamin/propanediol regulon of *Salmonella typhimurium*. *J. Bacteriol.* **175**:7200-7208.
- Aldrich, H. C., M. McDowell, F. S. de Barbosa, L. P. Yomano , R. K. Scopes, and L. O. Ingram.** 1992. Immunocytochemical localization of glycolytic and fermentative enzymes in *Zymomonas mobilis*. *J. Bacteriol.* **174**:4504-4508.
- Allen, R. H., S. P. Stabler, D. G. Savage, and J. Lindenbaum.** 1993. Metabolic abnormalities in cobalamin (vitamin B₁₂) folate deficiency. *FASEB J.* **7**:1344-1353.
- Allen, S. G. H., R. Kellermeyer, R. Stjernholm, B. Jacobson, and H. G. Wood.** 1963. The isolation, purification, and properties of methylmalonyl racemase. *J. Biol. Chem.* **238**:1637-1642.
- Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. *J. Mol. Biol.* **215**:403-410.
- Andersson, D. I.** 1992. Involvement of the Arc system in redox regulation of the *cob* operon in *Salmonella typhimurium*. *Mol. Microbiol.* **6**:1491-1494.
- Badger, M. R., and G. D. Price.** 1992. The CO₂ concentrating mechanism in cyanobacteria and microalgae. *Physiologia Plantarum* **84**:606-615.
- Badger, M. R., and G. D. Price.** 2003. CO₂ concentrating mechanisms in cyanobacteria: molecular components, their diversity and evolution. *J. Exp. Bot.* **54**:609-622.
- Baker, P. R., and K. R. Clauer.** <http://prospector.ucsf.edu>.
- Baker, S. H., S. C. Lorbach, M. Rodriguez-Buey, D. S. Williams, H. C. Aldrich, and J. M. Shively.** 1999. The correlation of the *csos2* gene of the carboxysome operon with two polypeptides of the carboxysome in *Thiobacillus neapolitanus*. *Arch. Microbiol.* **172**:233-239.

- Baker, S. H., D. S. Williams, H. C. Aldrich, A. C. Gambrell, and J. M. Shively.** 2000. Identification and localization of the carboxysome peptide Csos3 and its corresponding gene in *Thiobacillus neapolitanus*. *Arch. Microbiol.* **173**:278-283.
- Banerjee, R. V., and R. G. Matthews.** 1990. Cobalamin-dependent methionine synthase. *FASEB J.* **4**:1450-1459.
- Bassford Jr., P. J., and R. J. Kadner.** 1977. Genetic analysis of components involved in vitamin B₁₂ uptake in *Escherichia coli*. *J. Bacteriol.* **132**:796-805.
- Benner, S. A., A. D. Ellington, and A. Tauer.** 1989. Modern metabolism as a palimpsest of the RNA world. *Proc. Natl. Acad. Sci., USA* **86**:7054-7058.
- Berggren, K., E. Chernokalskaya, T. H. Steinberg, C. Kemper, M. F. Lopez, Z. Diwu, R. P. Haugland, and W. F. Patton.** 2000. Background-free, high sensitivity staining of proteins in one- and two-dimensional sodium dodecyl sulfate-polyacrylamide gels using a luminescent ruthenium complex. *Electrophoresis* **21**:2509-2521.
- Berkowitz, D., J. Hushon, H. Whitfield, Jr., J. Roth, and B. Ames.** 1968. Procedure for identifying nonsense mutations. *J. Bacteriol.* **96**:215-220.
- Birch, A., A. Leiser, and J. A. Robinson.** 1993. Cloning, sequencing, and expression of the gene encoding methylmalonyl-coenzyme A mutase from *Streptomyces cinnamonensis*. *J. Bacteriol.* **175**:3511-3519.
- Blakley, R.** 1965. Cobamides and ribonucleotide reduction. I. Cobamide stimulation of ribonucleotide reduction in extracts of *Lactobacillus leichmannii*. *J. Biol. Chem.* **240**:2173-2180.
- Blakley, R., and H. Barker.** 1964. Cobamide stimulation of the reduction of ribotides to deoxyribotides in *Lactobacillus leichmannii*. *Biochem. Biophys. Res. Commun.* **16**:391-397.
- Bobik, T. A., M. Ailion, and J. R. Roth.** 1992. A single regulatory gene integrates control of vitamin B₁₂ synthesis and propanediol degradation. *J. Bacteriol.* **174**:2253-2266.
- Bobik, T. A., G. D. Havemann, R. J. Busch, D. S. Williams, and H. C. Aldrich.** 1999. The propanediol utilization (*pdu*) operon of *Salmonella enterica* serovar typhimurium LT2 includes genes necessary for formation of polyhedral organelles involved in coenzyme B₁₂-dependent 1,2-propanediol degradation. *J. Bacteriol.* **181**:5967-5975.

- Bobik, T. A., Y. Xu, R. M. Jeter, K. E. Otto, and J. R. Roth.** 1997. Propanediol utilization genes (*pdu*) of *Salmonella typhimurium*: three genes for the propanediol dehydratase. *J. Bacteriol.* **179**:6633-6639.
- Bock, A., A. Priege-Kraft, and P. Schönheit.** 1994. Pyruvate: a novel substrate for growth and methane formation in *Methanosarcina barkeri*. *Arch. Microbiol.* **161**:33-46.
- Booker, S., S. Lichth, J. Broderick, and J. Stubbe.** 1994. Coenzyme B₁₂-dependent ribonucleotide reductase: evidence for the participation of five cysteine residues in ribonucleotide reduction. *Biochemistry* **33**:12676-12685.
- Bradbeer, C.** 1965. The clostridial fermentations of choline and ethanolamine. II. Requirement for a cobamide coenzyme by an ethanolamine deaminase. *J. Biol. Chem.* **240**:4675-4681.
- Bradbeer, C.** 1991. Cobalamin transport in *Escherichia coli*. *Biofactors* **3**:11-19.
- Bradbeer, C.** 1993. The proton motive force drives the outer membrane transport of cobalamin in *Escherichia coli*. *J. Bacteriol.* **175**:3146-3150.
- Bradbeer, C., J. S. Kenley, D. R. di Masi, and M. Leighton.** 1978. Transport of vitamin B₁₂ in *Escherichia coli*. Corrinoid specificities of the periplasmic B₁₂-binding protein and of energy-dependent B₁₂ transport. *J. Biol. Chem.* **253**:1347-1352.
- Bradbeer, C., and M. L. Woodrow.** 1976. Transport of vitamin B₁₂ in *Escherichia coli*: energy dependence. *J. Bacteriol.* **128**:99-104.
- Brown, F. B., J. C. Cain, D. E. Gant, F. J. Parker, and E. L. Smith.** 1955. The vitamin B₁₂ group. Presence of 2-methyl purines in factors *A* and *H* and isolation of new factors. *Biochem. J.* **59**:82-86.
- Bry, L., P. G. Falk, T. Midtvedt, and J. I. Gordon.** 1996. A model of host-microbial interactions in an open mammalian ecosystem. *Science* **273**:1380-1383.
- Burke, S., and J. Krzycki.** 1995. Involvement of the "A" isozyme of methyltransferase II and the 29-kilodalton corrinoid protein in methanogenesis from monomethylamine. *J. Bacteriol.* **177**:4410-4416.
- Cannon, G. C., S. H. Baker, F. Soyer, D. R. Johnson, C. E. Bradburne, J. L. Mehlman, P. S. Davies, Q. L. Jiang, S. Heinhorst, and J. M. Shively.** 2003. Organization of carboxysome genes in the thiobacilli. *Curr. Microbiol.* **46**:115-119.

- Cannon, G. C., C. E. Bradburne, H. C. Aldrich, S. H. Baker, S. Heinhorst, and J. M. Shively.** 2001. Microcompartments in prokaryotes: carboxysomes and related polyhedra. *Appl. Environ. Microbiol.* **67**:5351-5361.
- Cannon, G. C., S. Heinhorst, C. E. Bradburne, and J. M. Shively.** 2002. Carboxysome genomics: a status report. *Functional Plant Biology* **29**:175-182.
- Cannon, G. C., and J. M. Shively.** 1983. Characterization of a homogenous preparation of carboxysomes from *Thiobacillus neapolitanus*. *Arch. Microbiol.* **134**:52-59.
- Chan, R. K., D. Botstein, T. Watanabe, and Y. Ogata.** 1972. Specialized transduction of tetracycline resistance by phage P22 in *Salmonella typhimurium*. II. Properties of a high-frequency-transducing lysate. *Virology* **50**:883 - 898.
- Chen, P., M. Ailion, T. Bobik, G. Stormo, and J. Roth.** 1995. Five promoters integrate control of the *cob/pdu* regulon in *Salmonella typhimurium*. *J. Bacteriol.* **177**:5401-5410.
- Chen, P., D. Andersson, and J. R. Roth.** 1994. The control region of the *pdu/cob* regulon in *Salmonella typhimurium*. *J. Bacteriol.* **176**:5474-5482.
- Codd, G. A.** 1988. Carboxysomes and ribulose bisphosphate carboxylase/oxygenase. *Adv. Microbial Physiol.* **29**:115-164.
- Conner, C. P., D. M. Heithoff, S. M. Julio, R. L. Sinsheimer, and M. J. Mahan.** 1998. Differential patterns of acquired virulence genes distinguish *Salmonella* strains. *Proc. Natl. Acad. Sci. USA* **95**:4641-4645.
- Conway, T., G. W. Sewell, Y. A. Osman, and L. O. Ingram.** 1987. Cloning and sequencing of the alcohol dehydrogenase II gene from *Zymomonas mobilis*. *J. Bacteriol.* **169**:2591-2597.
- Datsenko, K. A., and B. L. Wanner.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. USA* **97**:6640-6645.
- Davis, R. W., D. Botstein, and J. R. Roth.** 1980. Advanced bacterial genetics : a manual for genetic engineering. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- DeVeaux, L. C., D. S. Clevenson, C. Bradbeer, and R. J. Kadner.** 1986. Identification of the BtuCED polypeptides and evidence for their role in vitamin B₁₂ transport in *Escherichia coli*. *J. Bacteriol.* **167**:920-927.
- DeVeaux, L. C., and R. J. Kadner.** 1985. Transport of vitamin B₁₂ in *Escherichia coli*: Cloning of the *btuCD* region. *J. Bacteriol.* **162**:888-896.

- Drennan, C. L., R. G. Matthews, and M. L. Ludwig.** 1994. Cobalamin-dependent methionine synthase: the structure of a methylcobalamin-binding fragment and implications for other B₁₂-dependent enzymes. *Curr. Opin. Struct. Biol.* **4**:919-929.
- Duda, J., Z. Pedziwilk, and K. Zodrow.** 1967. Studies on the vitamin B₁₂ content of the leguminous plants. *Acta Microbiol. Pol.* **6**:233-238.
- English, R. S., S. Jin, and J. M. Shively.** 1995. Use of electroporation to generate a *Thiobacillus neapolitanus* carboxysome mutant. *Appl. Environ. Microbiol.* **61**:3256-3260.
- English, R. S., S. C. Lorbach, X. Qin, and J. M. Shively.** 1994. Isolation and characterization of a carboxysome shell gene from *Thiobacillus neapolitanus*. *Mol. Microbiol.* **12**:647-654.
- Escalante, S. J. C., and J. R. Roth.** 1987. Regulation of cobalamin biosynthetic operons in *Salmonella typhimurium*. *J. Bacteriol.* **169**:2251-2258.
- Eschenmoser, A.** 1988. Vitamin B₁₂: experiments concerning the origin of its molecular structure. *Angew. Chem. Int. Ed. Eng.* **27**:5-39.
- Fantes, K. H., and O'Callaghan.** 1955. The effect of *o*-phenylenediamine on the biosynthesis of vitamin B₁₂: a new vitamin B₁₂ analogue. *Biochem. J.* **59**:79-82.
- Fenton, W. A., A. M. Hack, H. F. Willard, A. Gertler, and L. E. Rosenberg.** 1982. Purification and Properties of Methylmalonyl Coenzyme-a Mutase from Human-Liver. *Arch. Biochem. Biophys.* **214**:815-823.
- Ferry, J.** 1992. Methane from acetate. *J. Bacteriol.* **174**:5489-5495.
- Ferry, J.** 1993. Methanogenesis: ecology, physiology, biochemistry, and genetics. Chapman and Hall, New York.
- Ferry, J.** 1995. CO dehydrogenase. *Annu. Rev. Microbiol.* **49**:305-333.
- Flavin, M., and S. Ochoa.** 1957. Metabolism of Propionic Acid in Animal Tissues .1. Enzymatic Conversion of Propionate to Succinate. *J. Biol. Chem.* **229**:965-979.
- Ford, J. E., E. S. Holdsworth, and S. K. Kon.** 1955. The biosynthesis of B₁₂-like compounds. *Biochem. J.* **59**:86-93.
- Frey, B., J. McCloskey, W. Kersten, and H. Kersten.** 1988. New function of vitamin B₁₂: cobamide-dependent reduction of epoxyqueuosine to queuosine in tRNAs of *Escherichia coli* and *Salmonella typhimurium*. *J. Bacteriol.* **170**:2078-2082.

- Friedberg, D., A. Kaplan, R. Ariel, M. Kessel, and J. Seijffers.** 1989. The 5'-flanking region of the gene encoding the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase is crucial for growth of the cyanobacterium *Synechococcus* sp. strain PCC 7942 at the level of CO₂ in air. *J. Bacteriol.* **171**:6069-6076.
- Frings, J., and B. Schink.** 1994. Fermentation of phenoxyethanol to phenol and acetate by a homoacetogenic bacterium. *Arch. Microbiol.* **162**:199-204.
- Frings, J., E. Schramm, and B. Schink.** 1992. Enzymes involved in anaerobic polyethylene-glycol degradation by *Pelobacter venetianus* and *Bacteroides* strain PG1. *Appl. Environ. Microbiol.* **58**:2164-2167.
- Frings, J., C. Wondrak, and B. Schink.** 1994. Fermentative degradation of triethanolamine by a homoacetogenic bacterium. *Arch. Microbiol.* **162**:103-107.
- Guest, J. R., M. A. Foster, and S. Friedman.** 1962a. Alternative pathways for methylation of homocysteine by *Escherichia coli*. *Biochem. J.* **84**:93P-94P.
- Guest, J. R., E. L. Smith, D. D. Woods, and S. Friedman.** 1962b. A methyl analogue of cobamide coenzyme in relation to methionine synthesis by bacteria. *Nature* **195**:340-342.
- Harlow, E., and D. Lane.** 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Havemann, G. D., E. M. Sampson, and T. A. Bobik.** 2002. PduA is a shell protein of polyhedral organelles involved in coenzyme B₁₂-dependent degradation of 1,2-propanediol in *Salmonella enterica* serovar typhimurium LT2. *J. Bacteriol.* **184**:1253-1261.
- Heithoff, D. M., C. P. Conner, U. Hentschel, F. Govantes, P. C. Hanna, and M. J. Mahan.** 1999. Coordinate intracellular expression of *Salmonella* genes induced during infection. *J. Bacteriol.* **181**:799-807.
- Hodgkin, D. C., J. Pickworth, J. H. Robertson, K. N. Trueblood, R. J. Prosen, and J. G. White.** 1955. Crystal structure of the hexacarboxylic acid derived from B₁₂ and the molecular structure of the vitamin. *Nature* **176**:325-328.
- Hodgson, G. W., and C. Ponnamperuma.** 1968. Prebiotic porphyrin genesis: Porphyrins from electric discharge in methane, ammonia and water vapor. *Proc. Natl. Acad. Sci. USA* **59**:22-28.
- Holthuijzen, Y. A., J. F. L. van Breemen, J. G. Kurnen, and W. N. Konings.** 1986. Protein composition of the carboxysomes of *Thiobacillus neapolitanus*. *Arch. Microbiol.* **144**:398-404.

- Homann, T., C. Tag, H. Biebl, W. D. Deckwer, and B. Schink.** 1990. Fermentation of glycerol to 1,3-Propanediol by *Klebsiella* and *Citrobacter* Strains. *Appl. Microbiol. Biotechnol.* **33**:121-126.
- Horswill, A., and J. Escalante-Semerena.** 1997. Propionate catabolism in *Salmonella typhimurium* LT2: two divergently transcribed units comprise the *prp* locus at 8.5 centisomes, *prpR* encodes a member of the sigma-54 family of activators, and the *prpABCDE* genes constitute an operon. *J. Bacteriol.* **179**:928-940.
- Jensen, T. E., and C. C. Bowen.** 1961. Organization of the centroplasm of *Nostoc pruniforme*. *Proc. Iowa Acad. Sci.* **68**:89-96.
- Jeter, R. M.** 1990. Cobalamin-dependent 1,2-propanediol utilization by *Salmonella typhimurium*. *J. Gen. Microbiol.* **136**:887-896.
- Jeter, R. M., and J. R. Roth.** 1987. Cobalamin (vitamin B₁₂) biosynthetic genes of *Salmonella typhimurium*. *J. Bacteriol.* **169**:3189-3198.
- Johnson, C. L. V., E. Pechonick, S. D. Park, G. D. Havemann, N. A. Leal, and T. A. Bobik.** 2001. Functional genomic, biochemical, and genetic characterization of the *Salmonella pduO* gene, an ATP:cob(I)alamin adenosyltransferase gene. *J. Bacteriol.* **183**:1577-1584.
- Kadner, R. J., and G. L. Liggins.** 1973. Transport of vitamin B₁₂ in *Escherichia coli*: genetic studies. *J. Bacteriol.* **115**:514-521.
- Kaplan, A., and L. Reinhold.** 1999. CO₂ concentrating mechanisms in photosynthetic microorganisms. *Annu. Rev. Plant Physiol. and Plant Mol. Biol.* **50**:539-570.
- Kaplan, A., R. Schwarz, J. Liemanhurwitz, and L. Reinhold.** 1991. Physiological and molecular aspects of the inorganic carbon-concentrating mechanism in cyanobacteria. *Plant Physiol.* **97**:851-855.
- Kellermeyer, R. W., S. H. G. Allen, H. G. Wood, and R. Stjernholm.** 1964. Methylmalonyl isomerase .4. Purification and properties of enzyme from *Propionibacteria*. *J. Biol. Chem.* **239**:2562-2569.
- Keltjens, J., and G. Vogels.** 1994. Conversion of methanol and methylamines to methane and carbon dioxide. In J. Ferry (ed.), *Methanogenesis: ecology, physiology, biochemistry and genetics*. Chapman and Hall, New York.
- Kenley, J. S., M. Leighton, and C. Bradbeer.** 1978. Transport of vitamin B₁₂ in *Escherichia coli*. Corrinoid specificity of the outer membrane receptor. *J. Biol. Chem.* **253**:1341-1346.

- Lawrence, J. G., and J. R. Roth.** 1996. Evolution of coenzyme B₁₂ synthesis among enteric bacteria: evidence for loss and reacquisition of a multigene complex. *Genetics* **142**:11-24.
- Ledley, F.** 1990. Perspectives on methylmalonic acidemia resulting from molecular cloning of methylmalonyl CoA mutase. *Bioessays* **12**:335-340.
- Lenhert, P. G., and D. C. Hodgkin.** 1961. Structure of 5,6-dimethylbenzimidazolylcobamide coenzyme. *Nature* **192**:937-938.
- Lin, E. C. C.** 1987. Dissimilatory pathways for sugars, polyols, and carboxylates., p. 244-284. In F. D. Niedhardt, J. L. Ingraham, K. B. Low, et al. (ed.), *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*. American society for Microbiology, Washington, D.C.
- Ljungdahl, L., Wood H.** 1982. In B₁₂, ed. D Dolphin. 165-202.
- Ludwig, M., D. Sultemeyer, and G. D. Price.** 2000. Isolation of *ccmKLMN* genes from the marine cyanobacterium, *Synechococcus* sp. PCC7002 (Cyanophyceae), and evidence that CcmM is essential for carboxysome assembly. *Journal of Phycology* **36**:1109-1118.
- Ludwig, M. L., C. L. Drennan, and R. G. Matthews.** 1996. The reactivity of B₁₂ cofactors: the proteins make a difference. *Structure* **4**:505-512.
- Ludwig, M. L., and R. G. Matthews.** 1997. Structure-based perspectives on B₁₂-dependent enzymes. *Annu. Rev. Biochem.* **66**:269-313.
- Marco, E., I. Martinez, M. Ronen-Tarazi, M. I. Orus, and A. Kaplan.** 1994. Inactivation of *ccmO* in *Synechococcus* sp. strain PCC 7942 results in a mutant requiring high levels of CO₂. *Appl. Environ. Microbiol.* **60**:1018-1020.
- Marco, E., and M. I. Orus.** 1993. Trichlorfon-Induced Inhibition of Nitrate and Ammonium Uptake in Cyanobacteria. *Journal of Experimental Botany* **44**:501-508.
- McDowell, E. M., and B. F. Trump.** 1976. Histologic fixatives suitable for diagnostic light and electron microscopy. *Arch. Pathol. Lab. Med.* **100**:405-414.
- McGee, D. E., S. S. Carroll, M. W. Bond, and J. H. Richards.** 1982. Diol dehydratase: N-terminal amino acid sequences and subunit stoichiometry. *Biochem. Biophys. Res. Commun.* **108**:547-551.
- Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

- Miller, V. L., and J. J. Mekalanos.** 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. *J. Bacteriol.* **170**:2575-2583.
- Minot, G. R., and W. P. Murphy.** 1926. Treatment of pernicious anemia by a special diet. *J. Am. Med. Assoc.* **87**:470-476.
- Müller, G., K. Hlineny, E. Savvidis, F. Zipfel, J. Schiedl, and E. Schneider.** 1990. On the methylation process and cobalt insertion in cobyrinic acid biosynthesis, p. 281-298. In T. O. Baldwin (ed.), *Chemical aspects of enzyme biotechnology*. Plenum Press, New York.
- Müller, G., F. Zipfel, K. Hlineny, E. Savvidis, R. Hertle, and U. Traub-Eberhard.** 1991. Timing of cobalt insertion in vitamin B₁₂ biosynthesis. *J. Am. Chem. Soc.* **113**:9893-9895.
- Noguchi, S., Y. Nishimura, Y. Hirota, and S. Nishimura.** 1982. Isolation and characterization of an *E. coli* mutant lacking tRNA-guanine transglycosylase. Function and biosynthesis of queuosine tRNA. *J. Biol. Chem.* **257**:6544-6550.
- Obradors, N., J. Badía, L. Baldomà, and J. Aguilar.** 1988. Anaerobic metabolism of the L-rhamnose fermentation product 1,2-propanediol in *Salmonella typhimurium*. *J. Bacteriol.* **170**:2159-2162.
- Ogawa, T., D. Amichay, and M. Gurevitz.** 1994. Isolation and characterization of the *ccmM* gene required by the cyanobacterium *Synechocystis* PCC6803 for inorganic carbon utilization. *Photosynthesis Research* **39**:183-190.
- Ohkawa, H., M. Sonoda, H. Katoh, and T. Ogawa.** 1998. The use of mutants in the analysis of the CO₂-concentrating mechanism in cyanobacteria. *Can. J. Bot.* **76**:1035-1042.
- Ollagnier, S., E. Kervio, and J. Retey.** 1998. The role and source of 5'-deoxyadenosyl radical in a carbon skeleton rearrangement catalyzed by a plant enzyme. *FEBS Lett.* **437**:309-312.
- Orus, M. I., M. L. Rodriguez, F. Martinez, and E. Marco.** 1995. Biogenesis and ultrastructure of carboxysomes from wild type and mutants of *Synechococcus* sp. strain PCC7942. *Plant Physiol.* **107**:1159-1166.
- Overath, P., F. Lynen, E. R. Stadtman, and G. M. Kellerman.** 1962. Zum mechanismus der umlagerung von methylmalonyl-CoA in succinyl-CoA .3. Reinigung und eigenschaften der methylmalonyl-CoA-isomerase. *Biochemische Zeitschrift* **336**:77-98.

- Poirot, C., S. Kengen, E. Valk, J. Keltjens, C. van der Drift, and G. Vogels.** 1987. Formation of methylcoenzyme M from formaldehyde by cell free extracts of *Methanobacterium thermoautotrophicum*. Evidence for the involvement of a corrinoid-containing methyltransferase. FEMS Microbiol. Lett. **40**:7-13.
- Price, G. D., and M. R. Badger.** 1989. Expression of human carbonic-anhydrase in the cyanobacterium *Synechococcus* PCC7942 creates a high CO₂-requiring phenotype - Evidence for a central role for carboxysomes in the CO₂ concentrating mechanism. Plant Physiol. **91**:505-513.
- Price, G. D., and M. R. Badger.** 1989. Isolation and characterization of high CO₂-requiring-mutants of the cyanobacterium *Synechococcus* PCC7942 - Two phenotypes that accumulate inorganic carbon but are apparently unable to generate CO₂ within the carboxysome. Plant Physiol. **91**:514-525.
- Price, G. D., and M. R. Badger.** 1991. Evidence for the role of carboxysomes in the cyanobacterial CO₂-concentrating mechanism. Can. J. Bot. **69**:963-973.
- Price, G. D., S. M. Howitt, K. Harrison, and M. R. Badger.** 1993. Analysis of a genomic DNA region from the cyanobacterium *Synechococcus* sp. strain PCC7942 involved in carboxysome assembly and function. J. Bacteriol. **175**:2871-2879.
- Price, G. D., D. Sultemeyer, B. Klughammer, M. Ludwig, and M. R. Badger.** 1998. The functioning of the CO₂ concentrating mechanism in several cyanobacterial strains: a review of general physiological characteristics, genes, proteins, and recent advances. Can. J. Bot. **76**:973-1002.
- Price-Carter, M., J. Tingey, T. A. Bobik, and J. R. Roth.** 2001. The alternative electron acceptor tetrathionate supports B₁₂-dependent anaerobic growth of *Salmonella enterica* serovar typhimurium on ethanolamine or 1,2-propanediol. J. Bacteriol. **183**:2463-2475.
- Qureshi, A., D. Rosenblatt, and B. Cooper.** 1994. Inherited disorders of cobalamin metabolism. Crit. Rev. Oncol. Hematol. **17**:133-151.
- Ragsdale, S. W.** 1991. Enzymology of the acetyl-CoA pathway of CO₂ fixation. Crit. Rev. Biochem. Mol. Biol. **26**:261-300.
- Rapleye, C. A., and J. R. Roth.** 1997. A *Tn*10 derivative (T-POP) for isolation of insertions with conditional (tetracycline-dependent) phenotypes. J. Bacteriol. **179**:5827-5834.
- Refsum, H., P. M. Ueland, O. Nygard, and S. E. Vollset.** 1998. Homocysteine and cardiovascular disease. Annu. Rev. Med. **49**:31-62.

- Reichard, P.** 1993. From RNA to DNA, why so many reductases? *Science* **260**:1773-1777.
- Rickes, E. L., N. G. Brink, F. R. Koniuszy, T. R. Wood, and K. Folkers.** 1948. Crystalline vitamin B₁₂. *Science* **107**:396-397.
- Rondon, M. R., and J. Escalante-Semerena.** 1992. The *poc* locus is required for 1,2-propanediol-dependent transcription of the cobalamin biosynthetic (*cob*) and propanediol utilization (*pdu*) genes of *Salmonella typhimurium*. *J. Bacteriol.* **174**:2267-2272.
- Rondon, M. R., and J. C. Escalante-Semerena.** 1997. Integration host factor is required for 1,2-propanediol-dependent transcription of the *cob/pdu* regulon in *Salmonella typhimurium* LT2. *J. Bacteriol.* **179**:3797-3800.
- Rondon, M. R., R. Kazmierczak, and J. C. Escalante-Semerena.** 1995. Glutathione is required for maximal transcription of the cobalamin biosynthetic and 1,2-propanediol utilization (*cob/pdu*) regulon and for the catabolism of ethanolamine, 1,2-propanediol, and propionate in *Salmonella typhimurium*. *J. Bacteriol.* **177**:5434-5439.
- Roof, D. M., and J. R. Roth.** 1988. Ethanolamine utilization in *Salmonella typhimurium*. *J. Bacteriol.* **170**:3855-3863.
- Roth, J. R., J. G. Lawrence, and T. A. Bobik.** 1996. Cobalamin (coenzyme B₁₂): synthesis and biological significance. *Annu. Rev. Microbiol.* **50**:137-181.
- Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning : a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Sauvageot, N., C. Muller, A. Hartke, Y. Auffray, and J. M. Laplace.** 2002. Characterisation of the diol dehydratase *pdu* operon of *Lactobacillus collinoides*. *FEMS Microbiol. Lett.* **209**:69-74.
- Scarlett, F. A., and J. M. Turner.** 1976. Microbial metabolism of amino alcohols. Ethanolamine catabolism mediated by coenzyme B₁₂-dependent ethanolamine ammonia-lyase in *Escherichia coli* and *Klebsiella aerogenes*. *J. Gen. Microbiol.* **95**:173-176.
- Schmieger, H.** 1971. A method for detection of phage mutants with altered transducing ability. *Mol. Gen. Genet.* **110**:378-381.
- Schneider, Z.** 1987. Chemistry of cobalamin and related compounds. In Z. Schneider and A. Stroinski (ed.), *Comprehensive B₁₂*. Walter de Gruyter, Berlin.

- Schneider, Z., and A. Stroinski.** 1987a. Comprehensive B₁₂ : chemistry, biochemistry, nutrition, ecology, medicine. De Gruyter, Berlin ; New York.
- Schneider, Z., and A. Stroinski.** 1987b. Methylcobamide-dependent reactions, p. 259-266. In Z. Schneider and A. Stroinski (ed.), Comprehensive B₁₂. Walter de Gruyter, Berlin.
- Shively, J. M., F. Ball, D. H. Brown, and R. E. Saunders.** 1973a. Functional organelles in prokaryotes: polyhedral inclusions (carboxysomes) of *Thiobacillus neapolitanus*. Science **182**:584-586.
- Shively, J. M., L. F. Ball, and B. W. Kline.** 1973b. Electron microscopy of the carboxysomes (polyhedral bodies) of *Thiobacillus neapolitanus*. J. Bacteriol. **116**:1405-1411.
- Shively, J. M., C. E. Bradburne, H. C. Aldrich, T. A. Bobik, J. L. Mehiman, S. Jin, and S. H. Baker.** 1998. Sequence homologs of the carboxysomal polypeptide CsoS1 of the thiobacilli are present in cyanobacteria and enteric bacteria that form carboxysomes - polyhedral bodies. Can. J. Bot. **76**:906-916.
- Shively, J. M., G. L. Decker, and J. W. Greenawalt.** 1970. Comparative ultrastructure of the thiobacilli. J. Bacteriol. **101**:618-27.
- Shively, J. M., and R. S. English.** 1991. The carboxysome, a prokaryotic organelle: a mini-review. Can. J. Bot. **69**:957-962.
- Shively, J. M., G. van Keulen, and W. G. Meijer.** 1998. Something from almost nothing: carbon dioxide fixation in chemoautotrophs. Annu. Rev. Microbiol. **52**:191-230.
- Siri, P. W., P. Verhoef, and F. J. Kok.** 1998. Vitamins B₆, B₁₂, and folate: association with plasma total homocysteine and risk of coronary atherosclerosis. J. Am. Coll. Nutr. **17**:435-441.
- Smith, E. L., and L. F. J. Parker.** 1948. Purification of anti-pernicious anaemia factor. Biochem. J. **43**:R8-R9.
- Stojiljkovic, I., A. J. Baumler, and F. Heffron.** 1995. Ethanolamine utilization in *Salmonella Typhimurium*: nucleotide sequence, protein expression, and mutational analysis of the *cchA cchB eutE eutJ eutG eutH* gene cluster. J. Bacteriol. **177**:1357-1366.
- Stroinski, A.** 1987. Adenosylcobamide-dependent reactions, p. 226-259. In Z. Schneider and A. Stroinski (ed.), Comprehensive B₁₂. Walter de Gruyter, Berlin.

- Stupperich, E.** 1993. Recent advances in elucidation of biological corrinoid functions. *FEMS Microbiol. Rev.* **12**:349-365.
- Stupperich, E., H. J. Eisinger, and S. Schurr.** 1990. Corrinoids in Anaerobic-Bacteria. *FEMS Microbiol. Rev.* **87**:355-359.
- Stupperich, E., I. Steiner, and H. J. Eisinger.** 1987. Substitution of Co alpha-(5-hydroxybenzimidazolyl)cobamide (factor III) by vitamin B₁₂ in *Methanobacterium thermoautotrophicum*. *J. Bacteriol.* **169**:3076-81.
- Taylor, R. T., S. A. Norrell, and M. L. Hanna.** 1972. Uptake of cyanocobalamin by *Escherichia coli* B: Some characteristics and evidence for a binding protein. *Arch. Biochem. Biophys.* **148**:366-381.
- Textor, S., V. F. Wendisch, A. A. De Graaf, U. Muller, M. I. Linder, D. Linder, and W. Buckel.** 1997. Propionate oxidation in *Escherichia coli*: evidence for operation of a methylcitrate cycle in bacteria. *Arch. Microbiol.* **168**:428-436.
- Thauer, R., R. Hedderich, and R. Fischer.** 1993. Reactions and enzymes involved in methanogenesis from CO₂ and H₂, p. 209-252. In J. Ferry (ed.), *Methanogenesis: ecology, physiology, biochemistry, and genetics*. Chapman and Hall, New York.
- Toraya, T.** 2002. Enzymatic radical catalysis: coenzyme B12-dependent diol dehydratase. *Chem Rec* **2**:352-366.
- Toraya, T., and S. Fukui.** 1977. Immunochemical evidence for the difference between coenzyme-B₁₂-dependent diol dehydratase and glycerol dehydratase. *Eur. J. Biochem.* **76**:285-289.
- Toraya, T., S. Honda, and S. Fukui.** 1979. Fermentation of 1,2-propanediol and 1,2-ethanediol by some genera of *Enterobacteriaceae*, involving coenzyme B₁₂-dependent diol dehydratase. *J. Bacteriol.* **139**:39-47.
- Toraya, T., and K. Mori.** 1999. A reactivating factor for coenzyme B₁₂-dependent diol dehydratase. *J. Biol. Chem.* **274**:3372-3377.
- Toraya, T., K. Ushio, S. Fukui, and P. C. Hogenkamp.** 1977. Studies on the mechanism of the adenosylcobalamin-dependent diol dehydrase reaction by the use of analogs of the coenzyme. *J. Biol. Chem.* **252**:963-970.
- Tsang, A. W., A. R. Horswill, and J. C. Escalante-Semerena.** 1998. Studies of regulation of expression of the propionate (*prpBCDE*) operon provide insights into how *Salmonella typhimurium* LT2 integrates its 1,2-propanediol and propionate catabolic pathways. *J. Bacteriol.* **180**:6511-6518.

- Vogel, H. J., and D. M. Bonner.** 1956. Acetylornithinase of *Escherichia coli* - Partial purification and some properties. *J. Biol. Chem.* **218**:97-106.
- Walter, D., M. Ailion, and J. Roth.** 1997. Genetic characterization of the *pdu* operon: use of 1,2-propanediol in *Salmonella typhimurium*. *J. Bacteriol.* **179**:1013-1022.
- Way, J. C., M. A. Davis, D. Morisato, D. E. Roberts, and N. Kleckner.** 1984. New Tn10 derivatives for transposon mutagenesis and for construction of *lacZ* operon fusions by transposition. *Gene* **32**:369-379.
- White, J. C., P. M. di Girolamo, M. L. Fu, Y. A. Preston, and C. Bradbeer.** 1973. Transport of vitamin B₁₂ in *Escherichia coli*. Location and properties of the initial B₁₂-binding site. *J. Biol. Chem.* **248**:3976-3986.
- Witt, U., R. J. Muller, J. Augusta, H. Widdecke, and W. D. Deckwer.** 1994. Synthesis, properties and biodegradability of polyesters based on 1,3-propanediol. *Macromolecular Chemistry and Physics* **195**:793-802.
- Wood, H. G., S. W. Ragsdale, and E. Pezacka.** 1986. A new pathway of autotrophic growth utilizing carbon monoxide or carbon dioxide and hydrogen. *Biochem. Int.* **12**:421-440.
- Wu Tian, F., D. Liu Yong, and R. Song Li.** 2000. Selection and ultrastructural observation of a high-CO₂-requiring mutant of cyanobacterium *Synechococcus* sp. PCC7942. *Acta Botanica Sinica* **42**:116-121.
- Wu, W. F., M. L. Urbanowski, and G. V. Stauffer.** 1992. Role of the MetR regulatory system in vitamin B₁₂-mediated repression of the *Salmonella typhimurium metE* gene. *J. Bacteriol.* **174**:4833-4837.
- Zacharius, R. M., T. E. Zell, J. H. Morrison, and J. J. Woodlock.** 1969. Glycoprotein staining following electrophoresis on acrylamide gels. *Anal Biochem* **30**:148-152.
- Zhang, W. Z., and B. T. Chait.** 2000. Profound: An expert system for protein identification using mass spectrometric peptide mapping information. *Anal.Chem.* **72**:2482-2489.
- Zhu, Y., and E. C. Lin.** 1989. L-1,2-propanediol exits more rapidly than L-lactaldehyde from *Escherichia coli*. *J. Bacteriol.* **171**:862-867.

BIOGRAPHICAL SKETCH

Gregory D. Havemann was born and raised in Margate, Florida. He attended Margate Elementary and was accepted to the gifted program in third grade at which time he also took up playing the violin. At Margate Middle School he continued playing the violin and participated in the University of Central Florida Honors Orchestra in the summer of 1987. After middle school he attended the International Baccalaureate program at Boyd H. Anderson where he competed in cross-country. He obtained a Bachelor of Science degree in microbiology (with honors, along with a minor in chemistry) from the University of Florida in Gainesville, Florida in mid-1997. While there he conducted undergraduate research in the laboratory of Dr. Samuel Farrah studying the selective adhesion of bacteria to the minerals, dolomite and apatite. Also during his undergraduate studies, he held a part-time job under Dr. Edward Hoffman maintaining algae cultures and oysters for invertebrate immunology studies. Upon graduation, he began graduate studies at the University of Florida in the Department of Microbiology and Cell Science. There, under the mentoring of Dr. Thomas Bobik, he studied B₁₂-dependent metabolism in *Salmonella enterica*. He focused his research on organelles associated with the B₁₂-dependent degradation of 1,2-propanediol. He plans to continue his academic study as a postdoctoral research fellow.