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ABSTRACT

Andrea Jennifer McCoy, Ph.D. 2005

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Genetic and Biochemical Characterization of Peptidoglycan Synthesis in *Chlamydia*

The *Chlamydiaceae* family of bacteria are obligate, intracellular pathogens that cause significant diseases world-wide in both humans and animals. Despite having a cell envelope that resembles other gram-negative bacteria, the presence of peptidoglycan in the *Chlamydia* cell envelope has long been debated. Unlike other wall-less bacteria, chlamydiae synthesize penicillin-binding proteins, are sensitive to antibiotics that inhibit cell wall synthesis, and encode a nearly complete pathway for the synthesis of peptidoglycan. However, peptidoglycan has yet to be detected. In this work, the functionality of the peptidoglycan synthesis pathway in *C. trachomatis* was examined by genetically and biochemically characterizing key enzymes in the pathway.

Chapter 1 assesses the functionality of the chlamydial *murA* gene product as a UDP-*N*-acetylglucosamine enolpyruvyl transferase. MurA from *C. trachomatis* commits UDP-*N*-acetylglucosamine to the synthesis of peptidoglycan. The native chlamydial MurA contains an aspartate in the active site that confers resistance to fosfomycin, an antibiotic that inhibits MurA. Chapter 2.1 examines the D-alanyl-D-alanine ligase activity of the

unique chlamydial MurC-Ddl fusion protein when the chlamydial enzyme is expressed in *Escherichia coli*. Recombinant *C. trachomatis* MurC-Ddl catalyzes the specific ligation of two D-alanine. Furthermore, the D-alanyl-D-alanine activity of MurC-Ddl is the target of the antibiotic D-cycloserine. Chapter 2.2 examines the phenotypes observed when the chlamydial D-alanyl-D-alanine ligase is expressed in a D-alanyl-D-alanine requiring mutant of the facultative, intracellular pathogen *Shigella flexneri.* Finally, Chapter 3 summarizes attempts to identify the source of D-alanine for peptidoglycan synthesis in *Chlamydia.* In the absence of an apparent alanine racemase gene, the source of D-alanine for peptidoglycan synthesis in *Chlamydia* is assumed to be either the mammalian host or a chlamydial gene encoding racemase activity.

Taken together, the characterization of key enzymes in the PG synthesis pathway of *Chlamydia* suggests that these organisms synthesize PG and that the chlamydial PG structure is of the same composition as PG in other gram-negative bacteria. Furthermore, these findings pave the way for future research to answer the questions of how, when and why PG is synthesized in *Chlamydia.* The functionality of the PG synthesis pathway in *Chlamydia* opens the door to discovery of new and the use of pre-existing cell wall inhibitors for the treatment of chlamydial infections.

Genetic and Biochemical Characterization of Peptidoglycan Synthesis in *Chlamydia*

By

Andrea Jennifer McCoy

Dissertation submitted to the faculty of the Graduate Program in Molecular and Cell Biology of the Uniformed Services University of the Health Sciences

F. Edward Hébert School of Medicine

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Introduction

Chlamydiaceae

The *Chlamydiaceae* family of eubacteria are obligate intracellular bacteria that cause significant ocular, respiratory and sexually transmitted diseases in both humans and animals. Chlamydial infections are characterized by severe tissue damage and chronic infections presumably mediated by the induction of a persistent (cryptic) state by the host immune response (Beatty *et al.*, 1994). In humans, *Chlamydia trachomatis* is the primary cause of preventable infectious blindness (trachoma) worldwide in addition to being the leading cause of bacterial sexually transmitted infections. In women, genital infections caused by *C. trachomatis* are often asymptomatic and result in severe sequelae such as chronic pelvic inflammatory disease, ectopic pregnancy and involuntary sterility. An estimated 4-8 million new cases of *C. trachomatis* infections occur annually in the U.S. at a cost of $2-6 billion (Beagley and Timms, 2000). *C. pneumoniae* is a major human respiratory pathogen. Epidemiological surveillance has reported seroprevalence rates of 40-80% in the human population (Corsaro and Venditti, 2004). *C. pneumoniae* infections have also been linked to chronic diseases such as Alzheimer's, reactive arthritis and coronary heart disease, which is the most common cause of death of both men and women in the U.S. (Gilbert and Grayston, 2003). The remaining *Chlamydia* spp. (*C. muridarum, C. suis, C. psittaci, C. abortus, C. felis, C. caviae,* and *C. pecorum*) are veterinary pathogens although they can be transmitted to and cause infections in humans (Corsaro and Venditti, 2004). Animal infections of *C. suis, C. psittaci,* and *C. abortus* pose a severe economic impact on poulty and livestock industries.

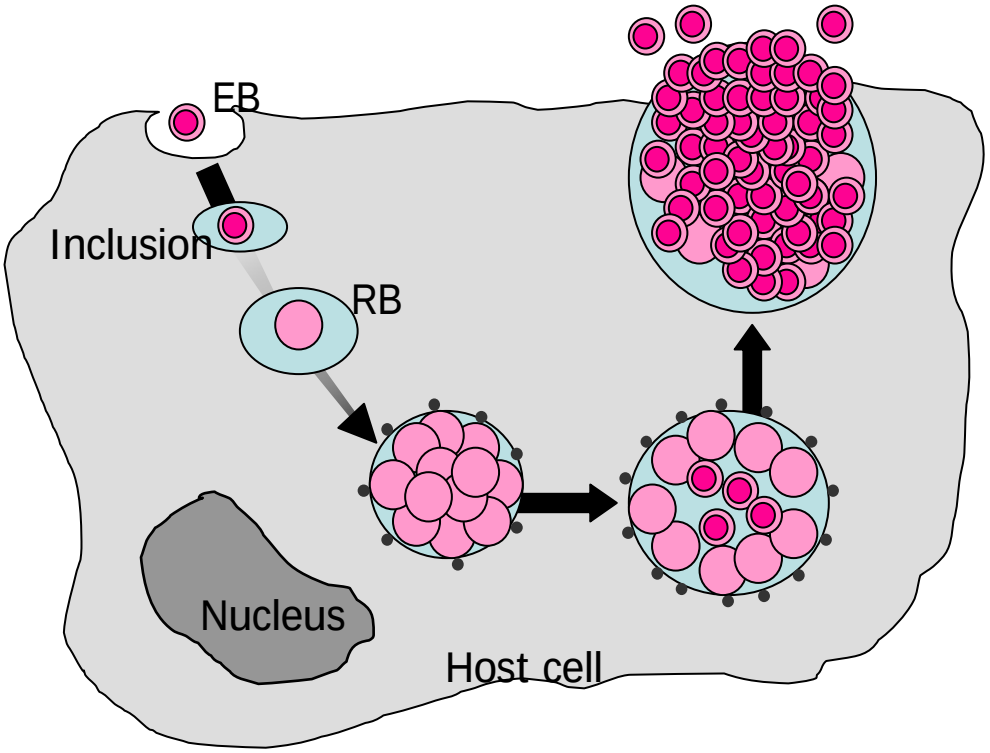
Chlamydiae possess a unique developmental cycle that oscillates between two morphologically distinct bodies (Figure I.1). Infectious but metabolically inactive elementary bodies (EBs) survive the extracellular environment to attach to and become internalized by host epithelial cells. Once inside the cell, *Chlamydia* resides in a specialized vacuole termed an inclusion throughout its life within the host cell. Shortly after internalization, the EB begins to differentiate into the non-infectious but metabolically active reticulate body (RB). After multiple rounds of division by binary fission, an unknown trigger event induces the RBs to re-differentiate back to EBs. Eventually (~36-72 hours post infection), the host cell lyses releasing a mixture of RBs, intermediate bodies and EBs, the latter of which can infect surrounding host cells and continue the developmental cycle.

Despite the public health and economic importance of *Chlamydia,* a more complete understanding of the unique biology of these bacteria has been limited due to the lack of a system of genetic manipulation. Fortunately for the chlamydial field, the difficulties imposed by the lack of a genetic manipulation system have been partially offset by the availability of the genome sequence of eight strains representing five chlamydial species (Read *et al.*, 2000; Read *et al.*, 2003; Shirai *et al.*, 2000; Stephens *et al.*, 1998; Thomson *et al.*, 2005). These genetic blueprints have provided new insights into chlamydial biology.

Peptidoglycan – General Characteristics

The cytoplasmic membrane of nearly all eubacteria is surrounded by a covalently closed, mesh-like polymer known as murein or peptidoglycan (PG). This macromolecular

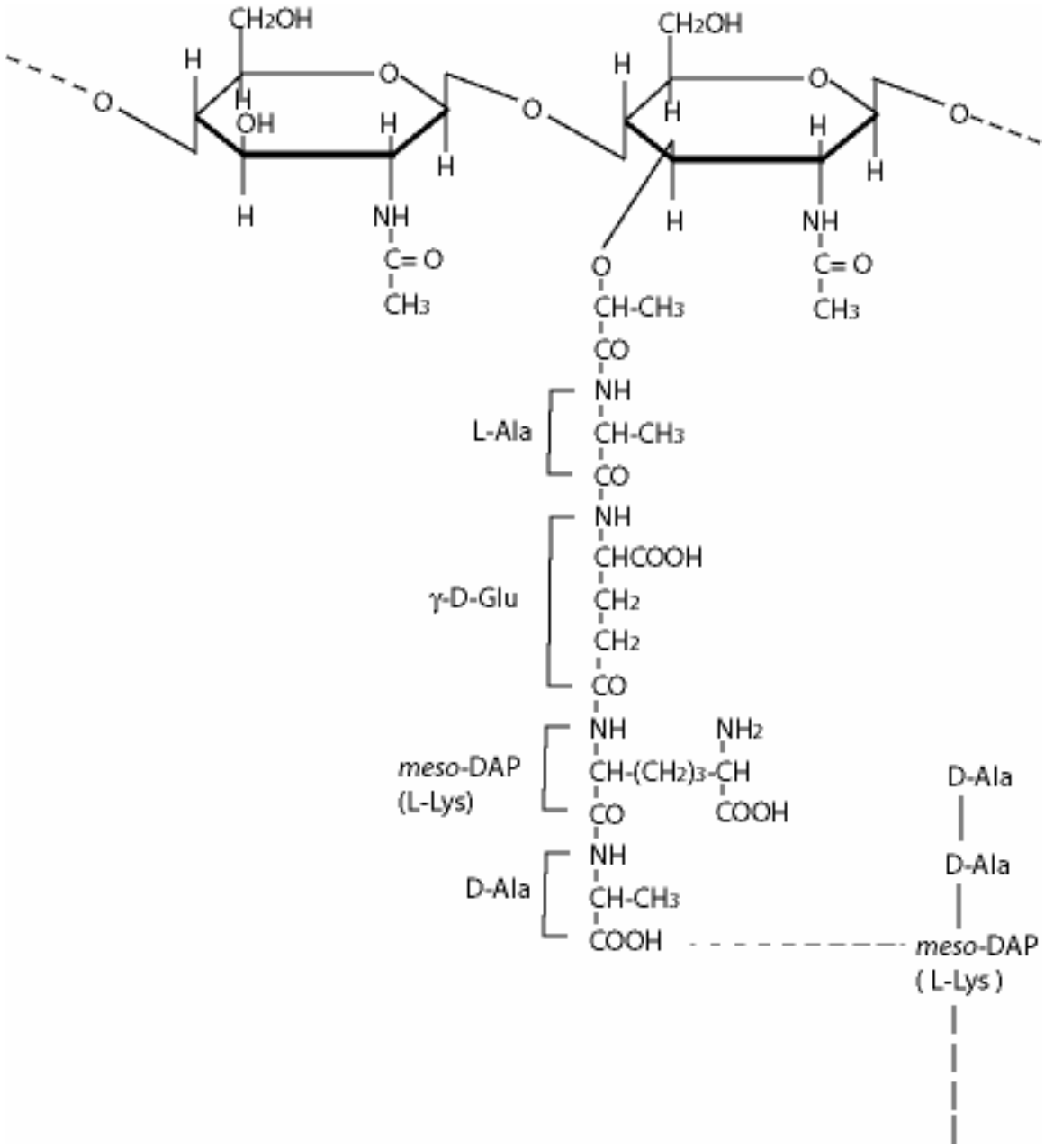
Figure I.1. The developmental cycle of *Chlamydia*. The developmental cycle of *Chlamydia* is initiated when an elementary body (EB) becomes internalized into a host mammalian cell. Inside the mammalian cell, *Chlamydia* resides within a specialized vacuole termed an inclusion. Within hours after internalization, the EBs begin to differentiate into reticulate bodies (RBs), which undergo numerous rounds of division. Eventually, the RBs begin to differentiate back to EBs and the mammalian cell lyses, releasing RBs, intermediate bodies and EBs. Released EBs can initiate another round of the developmental cycle within a surrounding cell.


EB
Inclusion
RB
Nucleus
Host cell

structure functions primarily to preserve the integrity of the bacterium under conditions of low external osmolarity although it also plays a significant role as a scaffold between daughter cells during cell division and in maintaining the morphology of the bacterium (Park, 1996). Bacterial cell walls are divided into two classes based upon their ability to retain crystal violet. gram-positive bacteria contain a thick, multi-layered PG structure that is embedded with teichoic acid and lipoteichoic acid. The embedded lipoteichoic acid also spans into the cytoplasmic membrane thereby anchoring the PG. In gram-negative bacteria, a single layer of PG is located in the periplasmic space between the cytoplasmic and outer membranes of the cell envelope and is covalently attached to the outer membrane via lipoproteins.

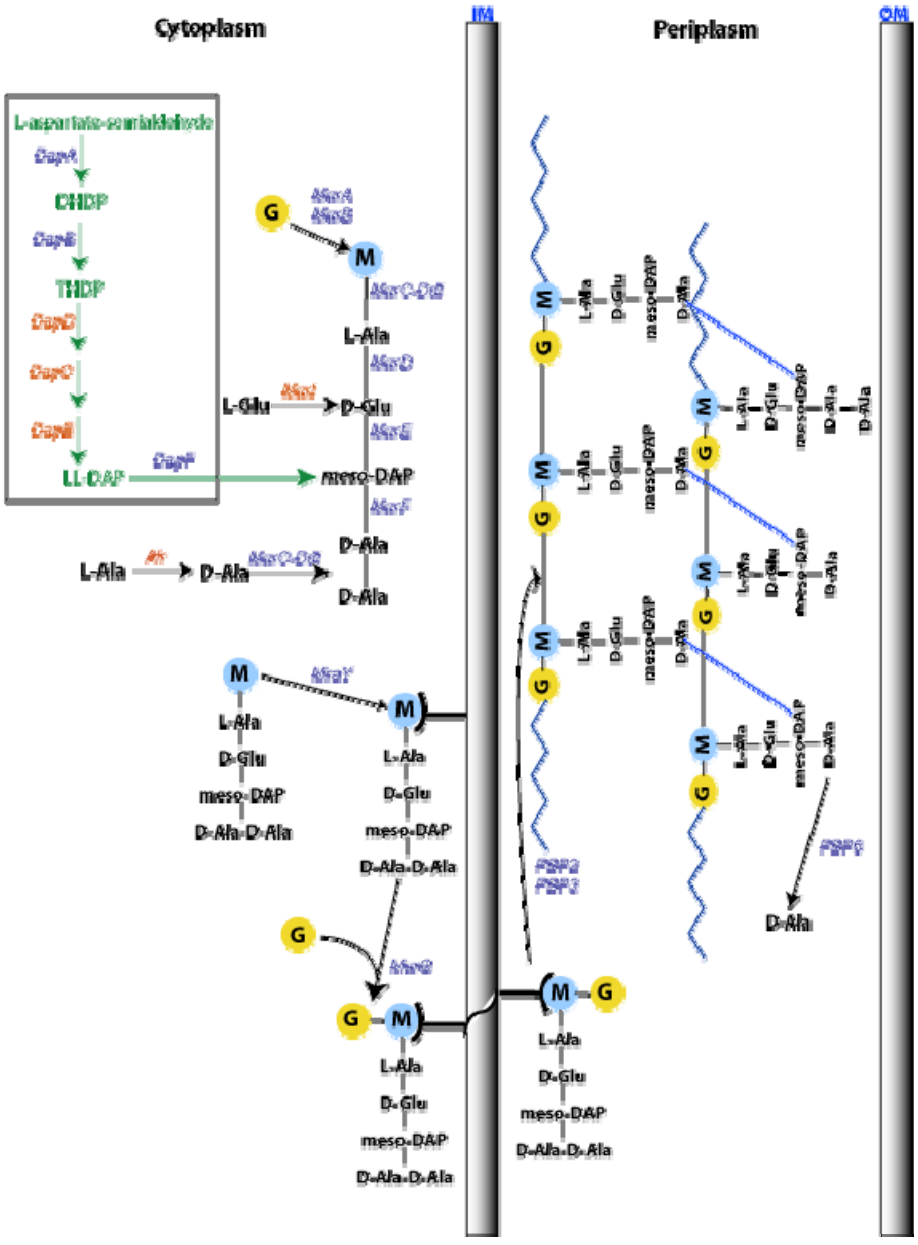
The PG structure consists of glycan chains of alternating β1→4 linked *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) (Figure I.2) (Park, 1996). Attached to MurNAc through an amide linkage to its carboxyl group is a peptide of the structure L-alanyl-D-glutamyl-*(a diamine)*-D-alanyl-D-alanine (L-Ala-D-Glu-(*X*)-D-Ala-D-Ala). In the gram-negative PG pentapeptide, the diamine (*X*) is *meso*-DAP while in the gram-positive structure, the diamine is often lysine. The diamine in the PG pentapeptide is required to cross-link the peptides. Cross-linking occurs between the penultimate D-Ala of one peptide and diamine of an adjacent peptide, which connects together the glycan chains. Often defined as inert, PG is actually a dynamic structure that is continuously remodeled in order to permit the bacterium to grow and divide. During this process PG fragments or muropeptides are also generated. Muropeptides act as potent biological effectors inducing cytokine production, autoimmunity and arthritis as well as exhibit potent cytotoxic effects on ciliated cells (Boneca, 2005).

Figure I.2. Chemical structure of PG. PG consists of interlocking chains of PG monomers. The PG monomer is composed of β1→4 linked *N*-acetylglucosamine and *N*-acetylmuramic acid. Attached to the *N*-acetylmuramic acid in the PG monomer is a pentapeptide. The short peptides are often cross-linked between the *meso*-DAP (or L-Lys) at position 3 of one PG monomer and the D-Ala at position 4 of an adjacent PG monomer, which results in the concomitant loss of the D-Ala at position 5. Cross-linking of the PG monomers can be direct or indirect via a peptide bridge.


CH2OH
H
O
H
OH
H
H
NH
C= O
CH3
O
CH-CH3
CO
NH
L-Ala
CH-CH3
CO
NH
CHCOOH
γ-D-Glu
CH2
CH2
CO
NH
NH2
meso-DAP
(L-Lys)
CH-(CH2)3-CH
CO
COOH
D-Ala
NH
D-Ala
CH-CH3
COOH
meso-DAP
(L-Lys)

The synthesis of PG (Figure I.3) has been studied for over half a decade, mostly in *Escherichia coli*, and has been extensively reviewed (Holtje, 1995; Holtje, 1996; van Heijenoort, 1996; Wong and Pompliano, 1998); (Bugg and Walsh, 1992; Cabeen and Jacobs-Wagner, 2005; Goffin and Ghuysen, 1998; Popham and Young, 2003; van Heijenoort, 2001, 2001). Synthesis occurs via a complex two-stage process. During the first stage (Stage I) PG monomers are assembled in the bacterial cytoplasm. Synthesis begins with the generation of the bacterial-specific nucleotide sugar, UDP-MurNAc, from UDP-GlcNAc via MurA and MurB. Next, amino acids are sequentially added to UDP-MurNAc by non-ribosomal synthesis proteins (MurC-F) ultimately giving rise to UDP-MurNAc-pentapeptide. After UDP-MurNAc-pentapeptide is synthesized, it is attached via MraY to an undecaprenol lipid carrier on the inner face of the cytoplasmic membrane thereby releasing the nucleotide. This is known as the Lipid I intermediate. MurG then adds UDP-GlcNAc to the Lipid I intermediate generating the Lipid II intermediate or the PG monomer. Finally, the PG monomer is translocated across the cytoplasmic membrane to the extracytoplasmic space. Although not considered part of the main pathway for PG synthesis, the enzymes involved in the formation of D-amino acids (D-Ala and D-Glu), D-Ala-D-Ala dipeptide and *meso*-DAP play supporting roles in PG synthesis as the assembly of the PG monomer is dependent upon these unusual amino acids. In the second stage (Stage II) of PG synthesis, the disaccharide PG monomers in the extracytoplasmic space are linked to the pre-existing glycan strand via glycosyltransferases while transpeptidases form the peptide bridges that link together the glycan strands.

Figure I.3. PG synthesis pathway. The synthesis of PG in gram-negative bacteria begins in the cytoplasm and terminates in the periplasm. The enzymes involved in the synthesis of PG (blue and red) have been highly conserved among PG containing bacteria. In *Chlamydia*, the PG synthesis pathway is nearly complete. Gene homologues encoding enzymes involved in the synthesis of D-Ala, D-Glu, and *meso*-DAP are missing (red). MurC and Ddl are encoded as a unique fusion protein in *Chlamydia.* **G** = *N*-acetylglucosamine; **M** = *N*-acetylmuramic acid; /\/\/\ = repeating PG monomer units; **DHDP** = dihydodipicolinate; **THDP** = tetrahydodipicolinate.


Cytoplasm
IM
Periplasm
OM
L-aspartate-semialdehyde
DapA
CHDP
DapB
THDP
DapD
DapC
DapE
LL-DAP
DapF
meso-DAP
L-Glu
Mur
D-Glu
MurE
L-Ala
MurC-C2
MurD
D-Ala
MurF
D-Ala
MurC-C2
G
MurA
MurB
M
MurG
PBP2
PBP3
PBP1
D-Ala

Stage I – Cytoplasmic assembly of the PG monomer

Synthesis of UDP-MurNAc

PG synthesis begins in the cytoplasm (Figure I.3) with the addition of an enolpyruvyl moiety from phosphoenolypyruvate (PEP) to UDP-GlcNAc. This addition is catalyzed by MurA (UDP-GlcNAc enolpyruvyl transferase) and commits UDP-GlcNAc to the synthesis of PG (Brown *et al.*, 1995). Mutational and structural analysis of MurA from both *E. coli* and *Enterobacter cloacae* have revealed that MurA folds into two similar globular domains while the catalytic site lies within a deep cavity between these domains (Schonbrunn *et al.*, 1998). Upon binding substrates, a solvent-exposed loop changes conformation such that it caps the catalytic cavity. This loop contains the critical Cys115 (*E. coli* numbering), which has been shown to be essential for product release (Eschenburg *et al.*, 2005). Besides Cys115, residues Lys22, Arg23 and Asp305 (*E. cloacae* numbering) are essential for enzyme activity and together with Arg91 these residues are highly conserved in MurA of different bacteria (Samland *et al.*, 1999; Samland *et al.*, 2001).

While UDP-GlcNAc can be used as a substrate in other bacterial pathways, the UDP-GlcNAc-enolpyruvate synthesized by MurA is specifically a substrate for MurB. MurB (UDP-GlcNAc enolpyruvyl reductase) is a flavoprotein that reduces the pyruvyl group of the UDP-GlcNAc-enolpyruvyl ether to D-lactate thereby generating UDP-MurNAc. MurNAc is the hallmark constituent of PG and is synthesized by bacteria exclusively for cell-wall synthesis. The structure of MurB from *E. coli* and *Staphylococcus aureus* has been solved and structural analysis has revealed that MurB folds into three distinct domains (Benson *et al.*, 2001). Domains 1 and 2 are involved in the binding of

FAD while domain 3 is responsible for the binding of NADPH and UDP-GlcNAc-enolpyruvate. Ser-229 has been shown to play a critical role in proton donation and enzymatic activity by mutational analysis. However, little work has been performed using mutational analysis to determine which amino acids in domains 1, 2, and 3 are critical for enzymatic activity.

Assembly of the MurNAc-pentapeptide

The PG pentapeptide is assembled by successive ligation of L-Ala, D-Glu, *meso*-DAP and D-Ala-D-Ala dipeptide to UDP-MurNAc by a group of related non-ribosomal synthesis proteins known as the Mur synthetases (MurC, MurD, MurE and MurF, respectively) (Figure I.2). These enzymes are functionally related in that they catalyze the formation of an amide or peptide bond with the concomitant hydrolysis of ATP to ADP and P_i (Bugg and Walsh, 1992; El Zoeiby *et al.*, 2003; van Heijenoort, 2001). Mur synthetases are also structurally related and share several conserved residues important for enzyme activity and enzyme structure (Bouhss *et al.*, 1997). These enzymes also contain an ATP-binding consensus sequence.

Mur synthetases from different organisms have been over-expressed, purified and studied kinetically (van Heijenoort, 2001). Besides L-Ala, MurC from *E. coli* and *Staphylococcus aureus* utilize glycine (Gly) and L-serine (L-Ser) as substrates *in vitro*. However, L-Ala is the preferred *in vivo* substrate in nearly all bacteria with the exception of *Corynebacterium* and *Mycobacterium* spp. In these organisms, Gly is the amino acid in position 1 of the PG pentapeptide. In contrast, MurD from *E. coli* is specific for D-Glu. The diamine-adding enzyme MurE is the most promiscuous in amino acid substrate

specificity of the Mur synthetases. *In vivo,* MurE from most gram-positive bacteria utilizes L-lysine as a substrate while in gram-negative bacteria, *meso*-DAP is the specific substrate of MurE. *In vitro* many different *meso*-DAP analogues, including LL-DAP, are substrates for the *E. coli* MurE. Furthermore, *meso*-DAP auxotrophic mutants of *E. coli* and mycobacteria are able to grow by incorporating L,L-DAP, cystathionine and lanthionine into the PG pentapeptide in place of *meso*-DAP (Consaul *et al.*, 2005). Interestingly, the PG pentapeptide of *Fusobacterium nucleatum* naturally contains lanthionine at the third position of the pentapeptide chain (Bugg and Walsh, 1992). In spite of the different amino acids that can be utilized as substrates by both MurC and MurE, the *E. coli* MurE enzyme displays a high specificity for UDP-MurNAc-L-Ala-D-Glu. The MurF enzyme is specific for dipeptides. This enzyme displays a high degree of specificity for dipeptides containing a C-terminal D-Ala and is less strict for an N-terminal D-Ala. This specificity is in sharp contrast to the specificity of the D-Ala-D-Ala ligase, which is highly specific for D-Ala in the N-terminal binding site of the enzyme (discussed below). Together, the substrate specificity of each of the Mur synthetase enzymes, as well as the D-Ala-D-Ala ligase, ensures the synthesis of MurNAc-L-Ala-D-Glu-*meso*-DAP-D-Ala-D-Ala in gram-negative bacteria.

Essential side reactions for PG synthesis

A. D-amino acids

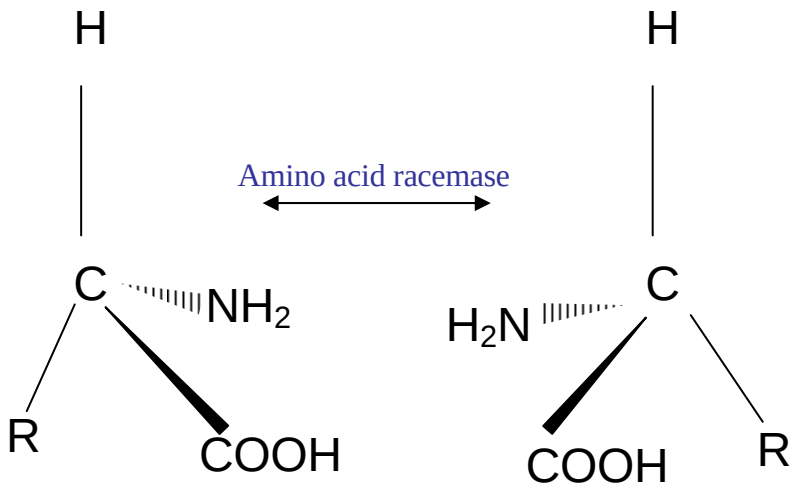
In nature, amino acids primarily occur as the L-isomer. PG is a relatively unique structure in that three of the five amino acids present in the PG-peptide are of the D-configuration, which presumably protects the cell wall from proteolytic degradation.

Hence, the enzymes involved in the formation of these amino acids are crucial to PG synthesis and the biology of the bacteria. Two mechanistic classes of enzymes exist for the synthesis of D-amino acids (Figure I.4) (Bugg and Walsh, 1992; van Heijenoort, 2001). Amino acid racemases catalyze the racemization of the α-carbon of an L-amino acid resulting in the respective D-amino acid. D-amino acid aminotransferases catalyze the reversible reduction of an α-keto acid to a D-amino acid.

In most bacteria, D-Glu and D-Ala are produced by glutamate and alanine racemases, respectively. Glutamate racemase is a member of a group of amino acid racemases and epimerases that function independently of a cofactor. For many bacteria, a single glutamate racemase is encoded in the genome. In *E. coli* the glutamate racemase gene *murI* is essential for the viability of the bacteria (van Heijenoort, 2001). *In vitro E. coli* MurI is activated by UDP-MurNAc-L-Ala suggesting that the glutamate racemase is activated post-translationally by PG synthesis possibly as a means of maintaining the L-Glu pool (van Heijenoort, 2001). Unlike glutamate racemase, alanine racemases require the cofactor pyridoxal-5-phosphate (PLP). PLP-dependent enzymes catalyze a broad range of reactions involved in amino acid metabolism (Schneider *et al.*, 2000). Two alanine racemase genes have been identified in *E. coli* (*alr* and *dadX*) and *Salmonella* (*alr* and *dadB*). The *alr* genes are constitutively expressed for the purpose of PG synthesis. In contrast, the *dadX/dadB* genes are inducibly expressed and serve to utilize L-Ala as a carbon source. In *E. coli* the loss of both *alr* and *dadX* is lethal to the bacteria (Strych *et al.*, 2001). In most bacteria, including *Lactobacillus* spp. and *Francisella,* only one alanine racemase gene (*alr*) is present in the genome and is essential for growth (Bron *et al.*, 2002; Gray *et al.*, 2002; Hols *et al.*, 1997; Tauch *et al.*, 2002).

Figure I.4. Mechanism of D-amino acid synthesis in bacteria. Bacteria utilize two mechanisms for the synthesis of D-amino acids.. Amino acid racemases (A) catalyze the conversion of L-amino acids to the D-enantiomer. L- and D-isomers are mirror images and are not superimposible. D-amino acid aminotransferases (B) catalyze the interconversion of various D-amino acids and 2-oxo acids and in most cases, the transamination is freely reversible. In (B), the donation of an amino group from D-Glu to pyruvate yields D-Ala and α-ketoglutarate.

A



B

D-amino acid aminotransferase

D-Glu + pyruvate ⇌ D-Ala + α-ketoglutarate

Both D-Glu and D-Ala are also produced via D-amino acid aminotransferases in certain gram-positive bacteria. *Bacillus* and *Staphylococcus* spp. possess a D-amino acid transaminase that yields D-Glu and pyruvate from D-Ala and α-ketoglutarate (Bugg and Walsh, 1992; van Heijenoort, 2001). The D-amino acid transaminase (*dat*) of *S. haemolyticus* and *B. sphaericus* is able to functionally complement D-Glu auxotrophy in an *E. coli murI* mutant (van Heijenoort, 2001). These bacteria also encode a glutamate racemase (*glr*) which can functionally complement an *E. coli* D-Glu auxotroph. It has been speculated that Glr may supply D-Glu for PG synthesis while the broader keto acid specificity of D-amino acid transaminases allow these bacteria to produce other D-amino acids (van Heijenoort, 2001). Bacilli are known to produce a number of D-amino acid containing antibiotics (Besson and Hourdou, 1987; Kuramitsu and Snoke, 1962; Zimmer, 1975).

Listeria monocytogenes encodes both an alanine racemase (*alr*) and a D-amino acid aminotransferase (*dat*). Dat catalyzes the reverse reaction of D-amino acid transaminases, *i. e.* D-Glu and pyruvate yield D-Ala and α-ketoglutarate. In the absence of exogenous D-Ala, *Listeria* mutants deleted of either gene are viable suggesting that both enzymes provide D-Ala (Thompson *et al.*, 1998). Deletion of both genes from the facultative intracellular *Listeria* renders the bacteria non-viable both extracellularly and intracellularly in the absence of exogenous D-Ala. The inability of *Francisella* and *Listeria* alanine racemase mutants to grow within mammalian cells suggests that insufficient concentrations of endogenous D-Ala exist within mammalian cells for the bacteria to utilize (Gray *et al.*, 2002).

B. D-Ala-D-Ala dipeptide

The D-Ala synthesized by alanine racemases and D-amino acid aminotransferases is a substrate for D-Ala-D-Ala ligases (Ddl). Ddl enzymes catalyze the ATP-dependent condensation of two D-Ala molecules. The resulting D-Ala-D-Ala dipeptide is the substrate of MurF during the assembly of the PG pentapeptide. Ddl proteins have two distinct D-amino acid binding sites (Bugg and Walsh, 1992; van Heijenoort, 2001). The N-terminal binding site displays a strict affinity for D-Ala while the C-terminal is more flexible in substrate specificity. Similar to alanine racemases, most bacteria encode only one Ddl homologue yet *E. coli* and *Salmonella* have two distinct *ddl* loci (*ddlA* and *ddlB*) in the genome. Despite only sharing approximately 35% amino acid identity to each other, DdlA and DdlB exhibit similar kinetic properties (Zawadzke *et al.*, 1991).

In vivo, D-Ala-D-Ala dipeptide is not the only substrate of MurF. The recent emergence of resistance to glycopeptide antibiotics such as vancomycin in *Enterococci* and *Staphylococci* has been attributed to the inducible incorporation of D-Ala-D-Lactate (D-Ala-D-Lac) and D-Ala-D-Serine (D-Ala-D-Ser) into the PG pentapeptide of these organisms (Evers *et al.*, 1996; Gholizadeh and Courvalin, 2000; Ruef, 2004; Witte, 2004). The formation of D-Ala-D-Lac and D-Ala-D-Ser is due to a family of enzymes known as the Van ligases (Evers *et al.*, 1996). These enzymes are structurally related to the Ddl ligases but display much broader substrate specificity. Extensive sequence alignment of Ddl and Van enzymes from different organisms reveals that the Van ligases share the conserved amino acids of the Ddl ligases (Evers *et al.*, 1996). Furthermore, the substrate specificity for the C-terminal amino acid is defined by a four amino acid stretch in these enzymes.

C. *meso*-DAP

In gram-negative bacteria, the synthesis of the unique diamine *meso*-DAP is also essential for PG synthesis. *meso*-DAP is synthesized in bacteria and plants as the precursor to L-lysine (Velasco *et al.*, 2002); (Hutton *et al.*, 2003). In mammalian cells, *meso*-DAP is neither synthesized by nor used as a substrate in any biosynthetic pathway (Saqib *et al.*, 1994). The first committed step in *meso*-DAP synthesis (Figure I.3) is the DapA-catalyzed condensation of L-aspartate-semialdehyde and pyruvate to generate dihydrodipicolinate (DHDP). DHDP is subsequently reduced by DapB to tetrahydrodipicolinate (THDP). From THDP, *meso*-DAP can be produced via three different branches (succinylase, acetylase, and dehydrogenase). The succinylase branch is the most predominantly utilized pathway in bacteria. In this branch, three reactions, catalyzed by DapD, DapC and DapE, convert THDP to L,L-DAP via succinylated intermediates. Similar to the succinylase branch, the acetylase branch also generates L,L-DAP via a three step reaction and acetylated intermediates. Ultimately, DapF epimerizes L,L-DAP to *meso*-DAP. In contrast, the dehydrogenase branch involves the direct conversion of THDP to *meso*-DAP via the DAP dehydrogenase, Ddh.

Stage II of PG synthesis – Assembly and cross-linking of glycan chains

After assembly of the Lipid II intermediate, the PG monomer is translocated from the inner face to the outer face of the cytoplasmic membrane. It is here that the second stage of PG assembly begins (Figure I.3). During this stage, the newly assembled disaccharide-pentapeptide monomers are incorporated into the pre-existing cell wall. In

order for this incorporation to occur, both polymerization events and restructuring of the closed covalent PG structure must take place.

The polymerization of PG occurs via two membrane-bound enzymatic processes, transglycosylation and transpeptidation. Transglycosylases catalyze the attachment of the disaccharides of the PG monomer to an existing PG glycan strand (Goffin and Ghuysen, 1998; van Heijenoort, 2001). Two types of glycosyltransferases, class A high molecular weight penicillin binding proteins (HMW-PBP) and monofunctional glycosyltransferases (Mgt), are involved in PG synthesis. The class A HMW-PBPs are multimodular proteins that possess a transmembrane spanner linked to the amino end of a non-penicillin-binding (n-PG) glycosyltransferase module which is linked to a peptidyltransferase penicillin-binding (PB) module. In contrast, Mgts share conserved amino acid sequences found in the class A HMW-PBP n-PB module but lack a PB module (Di Berardino *et al.*, 1996; Wang *et al.*, 2001).

Most bacterial species contain multiple glycosyltransferases for PG synthesis (van Heijenoort, 2001); (Goffin and Ghuysen, 1998). For many bacteria, loss of one or more of these proteins has little or no effect on the polymerization of PG or cell viability suggesting that these enzymes perform redundant functions (Denome *et al.*, 1999; McPherson *et al.*, 2001; McPherson and Popham, 2003; Popham and Setlow, 1994). However, essential functions have been shown for class A HMW-PBPs of both *E. coli* and *Streptococcus pneumoniae*. *E. coli* has one Mgt and three class A HMW-PBPs (PBP1a, PBP1b and PBP1c). All four glycosyltransferases polymerize PG monomers *in vitro* (van Heijenoort, 2001); (Goffin and Ghuysen, 1998). The loss of either PBP1a or PBP1b is not detrimental to *E. coli* however the loss of both is lethal even in the presence of PBP1c and Mgt

(Denome *et al.*, 1999; Yousif *et al.*, 1985). In *S. pneumoniae* the loss of PBP1a and PBP2a is lethal even in the presence of PBP1b and Mgt (Hoskins *et al.*, 1999). Surprisingly, the loss of all four identified glycosyltransferases in *B. subtilis* has little or no effect on the structure of the PG or the viability of the organism (McPherson and Popham, 2003). The presence of multiple glycosyltransferases in bacteria suggests that these enzymes may contribute to PG synthesis in different capacities. In support of this notion, PBP1b has been implicated in septal PG formation (Garcia-del Portillo and De Pedro, 1990).

After transglycosylation has occurred, PG peptides in adjacent glycan strands are cross-linked thereby linking together the glycan chains. These peptide linkages occur between the diamine at position 3 of one peptide and the D-Ala at position 4 of another peptide resulting in the concomitant loss of the terminal D-Ala (position 5) and are catalyzed by the peptidyltransferase activity of the PB module of the class A and class B HMW-PBPs (van Heijenoort, 2001); (Goffin and Ghuysen, 1998). Class B HMW-PBPs are structurally related to the class A HMW-PBPs in that they contain the membrane spanning, n-PB and PB modules. However, the class B n-PB module does not posses glycosyltransferase activity. Rather, the class B n-PB module is thought to interact with other proteins. The peptide linkages formed by the HMW-PBPs can either be direct, as in most gram-negative bacteria, or indirect via an interpeptide bridge. Occasionally, the formation of an L,D-peptide bond also occurs between two adjacent *meso*-DAP without the concomitant loss of the terminal D-Ala-D-Ala.

As discussed previously for the numerous glycosyltransferases in bacteria, the peptidyltransferase activity of the different HMW-PBPs likely play specialized roles within PG synthesis (Popham and Young, 2003; Spratt, 1975); (Cabeen and Jacobs-Wagner,

2005). Treatment of *E. coli* with β-lactams that specifically target PBP2 and FtsI (PBP3) leads to different outcomes for bacterial growth and development. Specific inhibition of PBP2 results in coccal-shaped *E. coli* suggesting a role in maintaining morphology. Localization studies support this role for PBP2 which appears to concentrate along the cylindrical wall of the cell. In contrast, specific inhibition of FtsI leads to long chains of bacteria that fail to septate suggesting a role for FtsI in cell division. Furthermore, FtsI is localized to the developing septa and completed poles, consistent with its primary role in cell division.

Modulation of the PG structure

Low molecular weight (LMW)-PBPs do not synthesize PG. Rather, these enzymes modify the PG structure by either removing the terminal D-Ala of PG pentapeptides (D,D-carboxypeptidases) or cleaving the D-Ala-*meso*-DAP linkage between cross-linked peptides (endopeptidases) (Goffin and Ghuysen, 1998; Popham and Young, 2003). *E. coli* encodes seven LMW-PBPs that can be subdivided into four enzymatic classes. PBP5, PBP6 and DacD are monofunctional D,D-carboxypeptidases; PBP4 is a bifunctional D,D-carboxypeptidase/endopeptidase; PBP7 is a monofunctional endopeptidase; and AmpC and AmpH are class C β-lactamases. Although the *in vitro* activities of the LMW-PBPs have been established, little is known about the *in vivo* functions of these enzymes.

To better understand the role(s) of the LMW-PBPs, Denome *et. al.* constructed mutants lacking all possible combinations of eight PBPs (Denome *et al.*, 1999). Surprisingly, an *E. coli* mutant deleted of the seven LMW-PBPs was viable and grew nearly as well as the parental strain suggesting that LMW-PBPs may not be essential under

laboratory growth conditions but rather under physiological conditions not yet tested. The function of only one LMW-PBP, PBP5, has been studied in depth. PBP5 mutants of *E. coli* exhibit slight morphological defects compared to the parent *E. coli* strain (Meberg *et al.*, 2004). However, when at least two other LMW-PBPs are deleted in the PBP5 mutant, these mutants are markedly more aberrant in cell diameter and contour (Nelson and Young, 2001). These defects cannot be complemented by another LMW-PBP suggesting that PBP5 plays a major role in maintaining the normal morphology of the cell. Recently, the endopeptidases PBP4 and PBP7 have been shown to work in concert with PBP5 to define the uniform morphology of *E. coli* possibly by acting as editing enzymes to remove inappropriate cross-links formed by PBP5 (Meberg *et al.*, 2004).

Macromolecular structure and assembly.

Despite the fact that the components the PG monomer and the enzymes that assemble PG are well characterized, the construction and higher order of the PG is not well understood as recently reviewed by Cabeen and Jacobs-Wagner (Cabeen and Jacobs-Wagner, 2005). Because stress is constantly exerted on the PG, insertion of new glycan strands into the pre-existing PG is challenging. Insertion of new material into the gram-positive cell wall occurs nearest to the cytoplasmic membrane. In gram-positive rods such as *B. subtilis,* new synthesis occurs along the sidewalls and division sites rather than at the cell poles. In spherical gram-positive organisms such as *S. aureus* and *Streptococcus* spp., new PG synthesis occurs predominantly at the sites of division. The newly inserted material is pushed outward into the thick multi-layered PG as PG synthesis continues.

Because the cell wall of gram-negative bacteria is mainly composed of a single layer of glycan strands, the insertion of new material is trickier and the mechanism has yet to be elucidated primarily due to the large number and variety of PG hydrolases with redundant functions (Cabeen and Jacobs-Wagner, 2005; Holtje and Heidrich, 2001). As in gram-positive rods, the insertion of new material in PG of gram-negative rods occurs primarily along the division sites as well as within the sidewall rather than at the poles. It can be reasoned that the activities of the glycosyltransferases, peptidyltransferases, and PG hydrolases must be tightly coordinated. Two distinct PBP complexes detected in *Haemophilus influenzae* support this notion (Alaedini and Day, 1999). Complex I contains the *E. coli* homologues PBP1a and FtsI (PBP3) while complex II contains the homologues PBP1b, PBP2 and PBP5. The molecular weights of the complexes are much greater than the sum of just the PBPs suggesting that other proteins are part of the complexes. FtsI (PBP3) is one of many Fts proteins recruited to the septal Z-ring during division as are the murein hydrolases AmiC and EnvC (Bernhardt and de Boer, 2003, 2004). Furthermore, the *E. coli* scaffolding protein MipA interacts with both PBP1b and MltA, a lytic transglycosylase, suggesting that cytoskeletal structural proteins like MipA, the tubulin-like homologue FtsZ, and the actin-like homologue MreB may influence the synthesis of PG (Vollmer *et al.*, 1999). However, further biochemical characterization of these complexes is needed.

PG and Stage I inhibitors

PG is an essential component of bacteria that is not found in higher organisms. This makes the enzymes in the PG synthesis pathway attractive targets for antimicrobial therapy. Two antibiotics, fosfomycin (FOS) and D-cycloserine (DCS), have been well

studied. Both antibiotics inhibit the activity of different enzymes involved in the assembly of the PG monomer. However, as with many antibiotics, the bacteria have adapted and become resistant to these PG inhibitors.

FOS is a naturally-occurring structural analogue of PEP produced by *Streptomyces.* FOS forms a covalent adduct with the Cys115 residue in the active site of MurA (Wong *et al.*, 2001). This interaction between MurA and FOS irreversibly inactivates MurA thereby terminating PG synthesis. Innate resistance to FOS has been demonstrated in *Mycobacteria* and can be attributed to a Cys115 to aspartate (Asp) alteration in MurA (De Smet *et al.*, 1999). Replacement of the Cys115 of the *E. coli* MurA with Asp renders the bacteria resistant to FOS, confirming this mechanism of FOS resistance (Kim *et al.*, 1996). Resistance to FOS is also attributed to decreased uptake of the drug and destroying the drug via a group of enzymes (FosA, FosB and FosX) that transfer glutathione, L-cysteine, and a hydroxyl group, respectively, to the ring of the antibiotic thereby rendering it inactive (Fillgrove *et al.*, 2003).

Alanine racemase and D-Ala-D-Ala ligases are inhibited by a few compounds, the most notable of which is D-cycloserine (DCS). DCS is a broad-spectrum, cyclic structural analogue of D-Ala that is produced by *Streptomyces garyphalus* and *Streptomyces lavendulae* and is used clinically for the treatment of tuberculosis. DCS irreversibly inactivates alanine racemases while reversibly inhibiting D-Ala-D-Ala ligases (Neuhaus and Hammes, 1981). Mechanisms of resistance to DCS include mutations that lead to increased expression of alanine racemases and/or D-Ala-D-Ala ligases and decreased uptake of the drug (Caceres *et al.*, 1997; Feng and Barletta, 2003; Wargel *et al.*, 1971).

Recently, a potential DCS efflux pump was identified in *Streptomyces garyphalus* and shown to confer DCS resistance to *E. coli* (Matsuo *et al.*, 2003).

Chlamydiaceae and peptidoglycan – the "chlamydial anomaly"

The cell envelope of *Chlamydia* resembles the envelopes of other gram-negative bacteria as they contain both an inner cytoplasmic and an outer membrane and lipopolysaccharide (LPS) is present in the outer membrane. However, unlike other gram-negative cell envelopes, a cell wall is not apparent in the *Chlamydia.* For many years the existence of PG in *Chlamydia* has been debated. Treatment of *Chlamydia*-infected cells with antibiotics that target cell-wall synthesis, such as β-lactams and DCS, leads to aberrant RB morphologies and the inhibition of RB division and transition from RB to EB (Chopra *et al.*, 1998; Moulder, 1993; Storey and Chopra, 2001). Penicillin-binding proteins have been detected in both EB and RB based upon their ability to bind radiolabeled penicillin (Barbour *et al.*, 1982; Chopra *et al.*, 1998). Nevertheless, PG has yet to be biochemically detected in *Chlamydia* (Chopra *et al.*, 1998; Moulder, 1993). Specifically, six reported studies have attempted to detect MurNAc or MurNAc derivatives in EBs while one attempt has been made using RBs**.** This contradiction has been termed "the chlamydial anomaly" (Moulder, 1993).

Because of the important role that PG plays in maintaining the osmotic stability of bacteria, its absence in *Chlamydia* is reconciled by the existence of cysteine-rich proteins in the chlamydial cell envelope (Hatch, 1996; Moulder, 1993). EBs are osmotically stable presumably due to the high degree of disulfide cross-linking of these cysteine-rich cell envelope proteins. In contrast, the disulfide bonds of these membrane proteins are reduced

in the osmotically fragile RBs. While the *Chlamydia* inclusion may provide an osmoprotective environment to the RBs, the notion that RBs are PG-less cannot account for the RB phenotypes observed when *Chlamydia* is treated with inhibitors of PG synthesis.

When the first chlamydial genome sequence data were published in 1998, it was revealed that *C. trachomatis* encoded a nearly complete pathway for the synthesis of PG. Currently, the genomes of eight strains representing five species of *Chlamydia* have been sequenced and in all, a nearly complete PG pathway is present (Read *et al.*, 2000; Read *et al.*, 2003; Shirai *et al.*, 2000; Stephens *et al.*, 1998; Thomson *et al.*, 2005). Compared to the *E. coli* PG synthesis pathway, three key features are missing from the *Chlamydiac* PG synthesis pathways. First, despite encoding a D-Ala-D-Ala ligase, no alanine racemase or even glutamate racemase is present in the genome suggesting that a PG structure in *Chlamydia* may be devoid of D-amino acids. Second, no glycosyltransferases for the assembly of the PG have been identified. This finding lead Ghuysen and Goffin to propose that a PG structure in *Chlamydia* is glycan-less as the PG monomers would only be linked between peptides (Ghuysen and Goffin, 1999). Finally, the *meso*-DAP synthesis pathway is incomplete as none of the branches of THDP to L,L-DAP/*meso*-DAP are present suggesting that a diamine other than *meso*-DAP occupies position 3 of the PG pentapeptide. One interesting feature of the chlamydial PG synthesis pathway is the fusion of the *murC* and *ddl* genes. The resulting product of this unique gene fusion is a protein fusion of the L-Ala adding enzyme and the D-Ala-D-Ala ligase. Of the over 200 bacterial genomes sequenced to date, only in the *Chlamydiaceae* family is this unique fusion found.

The discovery of this pathway in the *Chlamydiaceae* shed new light on the PG anomaly and raised even more questions. Are the genes non-functional remnants of an

ancestor or are they expressed in *Chlamydia*? At what stage of the developmental cycle are they expressed? Why did *Chlamydia* maintain this biosynthetic pathway if not to make PG? If the genes are expressed and translated, are the enzymes functional in synthesizing PG? Does the PG contain MurNAc or a unique sugar? Is the PG glycan-less or do the PG monomers assemble into glycan chains? Does the PG contain D-amino acids or *meso*-DAP? In this work, three objectives were established in order to begin to address some of these questions:

Specific Aim 1. To genetically and biochemically characterize the *C. trachomatis* MurA, the enzyme that commits UDP-GlcNAc to the synthesis of PG.

Specific Aim 2. To genetically and biochemically characterize the D-Ala-D-Ala ligase activity of the unique MurC-Ddl fusion in *C. trachomatis.*

Specific Aim 3. To identify and characterize an alanine racemase in *C. trachomatis*.

Chapter 1

IN VITRO AND IN VIVO FUNCTIONAL ACTIVITY OF *CHLAMYDIA* MURA, A UDP-N-ACETYLGLUCOSAMINE ENOLPYRUVYL TRANSFERASE INVOLVED IN PEPTIDOGLYCAN SYNTHESIS AND FOSFOMYCIN RESISTANCE

ABSTRACT

Chlamydia spp. are obligate intracellular, gram-negative bacteria with a dimorphic life cycle that takes place entirely within a membrane-bound vacuole termed an inclusion. The chlamydial anomaly refers to the fact that cell wall-active antibiotics inhibit *Chlamydia* growth and peptidoglycan synthesis genes have been identified in the genome, yet there is no biochemical evidence for synthesis of peptidoglycan. In this work, we undertook a genetics-based approach to re-evaluate the chlamydial anomaly by characterizing MurA, a UDP-*N*-acetylglucosamine enolpyruvyl transferase that catalyzes the first committed step of peptidoglycan synthesis. The *murA* gene from *C. trachomatis* serovar L2 was cloned and placed under the control of the arabinose-inducible, glucose-repressible *ara* promoter and transformed into *Escherichia coli*. After transduction of a lethal Δ*murA* mutation into the strain, viability of the *E. coli* became dependent upon expression of the *C. trachomatis murA.* DNA sequence analysis of *murA* from *C. trachomatis* predicted a cysteine to aspartate change in a key residue within the active site of MurA. In *E. coli,* the same mutation has been shown previously to cause resistance to fosfomycin (FOS), a potent antibiotic that specifically targets MurA. *In vitro* activity of the chlamydial MurA was

resistant to high levels of FOS. Growth of *C. trachomatis* was also resistant to FOS. Moreover, FOS resistance was imparted to the *E. coli* strain expressing the chlamydial *murA*. Conversion of *C. trachomatis* elementary bodies to reticulate bodies and cell division are correlated with expression of *murA* mRNA. mRNA from *murB*, the second enzymatic reaction in the peptidoglycan pathway, was also detected during *C. trachomatis* infection. Our findings, as well as work from other groups, suggest that a functional peptidoglycan pathway exists in *Chlamydia*. We propose that chlamydial peptidoglycan is essential for progression through the developmental cycle as well as for cell division. Elucidating the existence of peptidoglycan in *Chlamydia* is of significance for the development of novel antibiotics targeting the chlamydial cell wall.

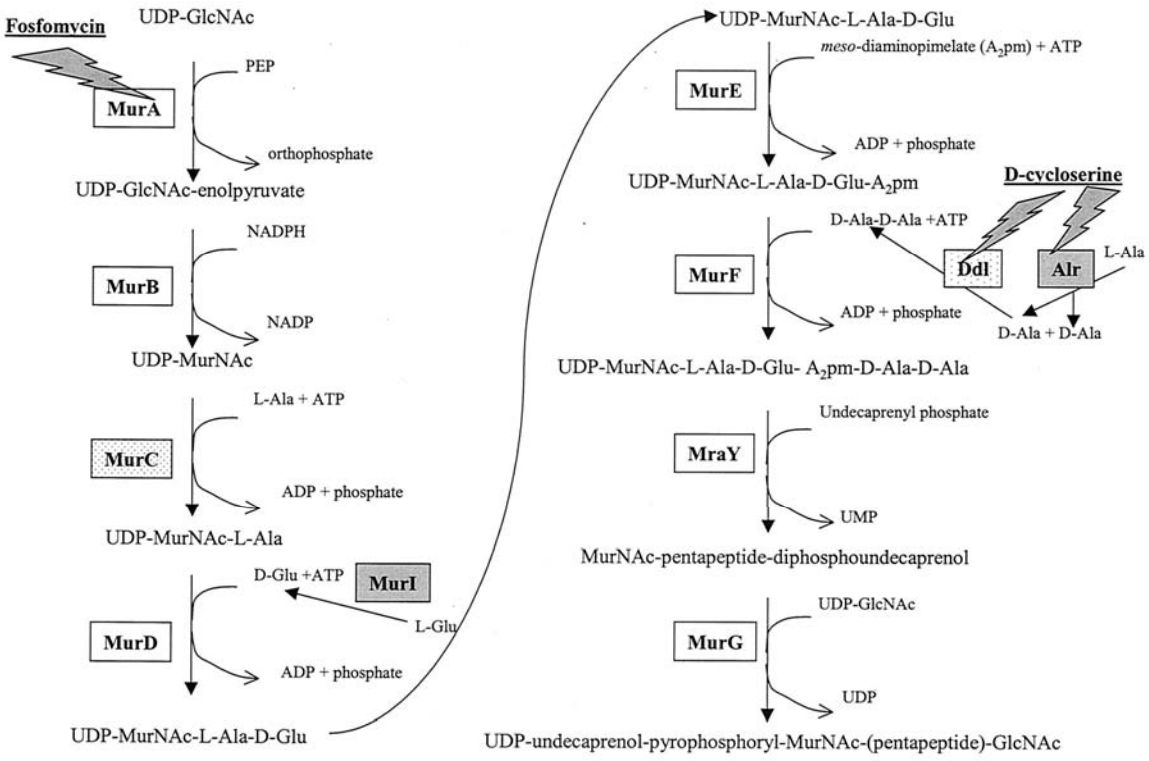
INTRODUCTION

Chlamydiae, of which there are four species, are gram-negative, obligate intracellular eubacteria. *Chlamydia psittaci* and *Chlamydia pecorum* are important animal pathogens while *Chlamydia trachomatis* and *Chlamydia pneumoniae* are important human pathogens that cause sexually transmitted, ocular and respiratory infections. Chlamydiae have a unique biphasic developmental cycle that allows the bacteria to survive in two different habitats (Wyrick, 2000). The infectious elementary bodies (EB) are small, osmotically stable, metabolically inactive particles that survive in the extracellular environment to attach to host epithelial cells and become internalized. Once inside, the bacteria reside in a specialized phagosome in the host cell cytoplasm, termed the chlamydial inclusion. In the inclusion the EBs differentiate into large, osmotically fragile, metabolically active reticulate bodies (RB), which use host cell ATP and nutrients and divide by binary fission. The cycle remains synchronous for approximately the first 20 hours post infection (hpi), after which some RBs continue to divide while others begin to differentiate back to EBs. The host cells continue to support growth of the bacteria (30 to 72 hpi) before the cells lyse, releasing EBs, RBs, and intermediate forms. Released EBs initiate subsequent rounds of infection in surrounding cells.

In addition to the unique developmental cycle, the chlamydial cell envelope differs from other eubacteria. Chlamydiae are classified as gram-negative bacteria as cell wall lipid and amino acid compositions are similar to other gram-negative organisms, and lipopolysaccharide is detected in the outer membrane (Raulston, 1995). However, unlike most gram-negative as well as gram-positive bacteria, chlamydiae do not contain detectable levels of peptidoglycan (PG). PG is a polymer of alternating *N*-acetylglucosamine

(GlcNAc) and *N*-acetylmuramic acid (MurNAc) units with an L- and D-amino acid-containing pentapeptide linked to MurNAc (Park, 1996). This polymer layer determines the shape of the bacteria and protects the bacteria from osmotic shock. PG also plays a role in cell division. Several observations support the notion that chlamydiae lack PG. These include the inability to detect a PG layer by electron microscopy, the failure of antibodies against PG to react with chlamydiae, and the inability to detect muramic acid, a marker for PG, using gas-chromatography-spectrometry (Chopra *et al.*, 1998; Fox *et al.*, 1990). Disulfide cross-linking of the chlamydial major outer membrane protein (MOMP) and cysteine-rich proteins in the outer membrane of EBs are believed to substitute for PG and provide structural strength to the cell wall (Hatch, 1996). Paradoxically, unlike other bacteria that lack PG, chlamydiae contain penicillin-binding proteins and are sensitive to antibiotics that inhibit PG synthesis (Barbour *et al.*, 1982; Moulder, 1993). Matsumoto and Manire demonstrated by transmission electron microscopy that penicillin treatment of *C. psittaci*-infected L cells prevented RB binary fission and resulted in the formation of abnormally large RBs (Matsumoto and Manire, 1970). These "penicillin forms", which resume normal shape after penicillin removal, have been reported in every species of *Chlamydia.* Lin and Moulder reported similar observations of *C. psittaci*-infected cells treated with D-cycloserine (Lin and Moulder, 1966). Correspondingly, genome sequencing of *C. trachomatis* serovars D and L2 and two strains of *C. pneumoniae* has revealed that both species contain a nearly complete pathway for PG synthesis, similar to the pathway in *Escherichia coli* (Figure 1.1). The paradox of phenotypic and genomic evidence for PG in *Chlamydia* and the absence of detectable PG has been termed the chlamydial anomaly (Moulder, 1993).

Figure 1.1. Pathway of lipid carrier-linked GlcNAc-MurNAc-pentapeptide in *E. coli* and *Chlamydia*. In the cytoplasm of *E. coli*, eleven enzymes (MurA-MurG, MurI, MraY, Alr, and Ddl) catalyze the synthesis of the disaccharide-pentapeptide subunit of PG. Of these eleven enzymes, *Chlamydia* encode nine. Alr and MurI (shaded gray) are missing. MurC and Ddl (speckled gray) are encoded as a fused protein in *Chlamydia.* Antibiotics that inhibit cell wall synthesis are underlined while gray bolts identify the enzymes targeted.


Fosfomycin
UDP-GlcNAc
PEP
MurA
orthophosphate
UDP-GlcNAc-enolpyruvate
NADPH
MurB
NADP
UDP-MurNAc
L-Ala + ATP
MurC
ADP + phosphate
UDP-MurNAc-L-Ala
D-Glu +ATP
MurI
L-Glu
MurD
ADP + phosphate
UDP-MurNAc-L-Ala-D-Glu
UDP-MurNAc-L-Ala-D-Glu
meso-diaminopimelate (A2pm) + ATP
MurE
ADP + phosphate
UDP-MurNAc-L-Ala-D-Glu-A2pm
D-cycloserine
D-Ala-D-Ala +ATP
MurF
Ddl
Alr
L-Ala
ADP + phosphate
D-Ala + D-Ala
UDP-MurNAc-L-Ala-D-Glu- A2pm-D-Ala-D-Ala
Undecaprenyl phosphate
MraY
UMP
MurNAc-pentapeptide-diphosphoundecaprenol
UDP-GlcNAc
MurG
UDP
UDP-undecaprenol-pyrophosphoryl-MurNAc-(pentapeptide)-GlcNAc

To date, there have been no reports demonstrating the functionality of an enzyme in the chlamydial PG pathway. We decided to take a genetic approach to demonstrate the functionality of the chlamydial MurA, the enzyme that commits UDP-GlcNAc to PG synthesis. MurA, a UDP-*N*-acetylglucosamine enolpyruvyl transferase, catalyzes the addition of enolpyruvate from phosphoenolypyruvate (PEP) to UDP-GlcNAc. In *E. coli*, *murA* is an essential gene. A single copy of *murA* exists in the genome and deletion of this gene is lethal (Brown *et al.*, 1995). MurA is also the site of action for the antibiotic FOS, a structural analog of PEP that binds irreversibly to the active site of MurA (Kahan *et al.*, 1974).

Unlike most other bacteria of clinical significance, a system for genetic manipulation of *Chlamydia* is not available. Characterization of chlamydial genes is thus far limited to expression in a heterologous host system such as *E. coli*. In this report, we show that MurA from *C. trachomatis* functions as a UDP-*N*-acetylglucosamine enolpyruvyl transferase by complementation of a lethal deletion of the essential *murA* gene in *E. coli*. Genome sequencing revealed that the chlamydial MurA contains a cysteine to aspartate change in the active site of the enzyme. A similar change in the *E. coli* MurA renders the enzyme resistant to FOS. We also demonstrate that *C. trachomatis* is resistant to high concentrations of FOS. This innate resistance to FOS appears to be due to the activity of the chlamydial MurA as *in vitro* activity of the chlamydial MurA is resistant to high concentrations of FOS and FOS resistance can also be conferred upon *E. coli* expressing the chlamydial MurA. *C. trachomatis* temporally express *murA* mRNA and mRNA from other PG pathway genes. Collectively, our data support the notion that

Chlamydia contain PG and suggest that PG in *Chlamydia* plays a role in RB development and division.

MATERIAL AND METHODS

Bacterial strains and growth conditions. Strains used in this study are listed in Table 1.1. Strains were grown at 37ºC in Luria-Bertani (LB) medium with aeration or on LB agar. When appropriate, medium was supplemented with ampicillin (100 μg/ml), kanamycin (50 μg/ml), chloramphenicol (15 μg/ml), arabinose (0.1%) or glucose (0.2%). FOS was obtained from Sigma Chemical Company (St. Louis, MO).

pAJM5 and pAJM6 contain the –10 and coding sequence of the *murA* gene from *E. coli* K-12 and *C. trachomatis* serovar L2, respectively, under the control of the *ara* promoter of the pBAD vectors. pBAD was chosen for its arabinose-inducible glucose-repressible regulation of gene expression. The *E. coli murA* gene was released from pBAD30-Z (Brown *et al.*, 1995) as a 1.4 kb *Xba*I-*Kpn*I fragment and ligated into *Xba*I-*Kpn*I-digested pBAD33. This plasmid was then digested with *Sac*I-*Sph*I and the liberated *murA* fragment was ligated into *Sac*I-*Sph*I-digested pBAD18 to create pAJM5. The chlamydial *murA* was PCR amplified from *C. trachomatis* serovar L2 genomic DNA using *Taq* polymerase (Qiagen, Valencia, CA). The upstream primer (5'-CGGAATTCGAAGGAACTGAA**ATG**CCTG-3') contained an *Eco*RI restriction site (underlined) and the –10 region and the start codon (bold), whereas the downstream primer (5'-GCGAATTCT**TAA**ACATACACAGATAC-3') contained an *Eco*RI restriction site (underlined) and the stop codon (bold). Both primers were designed based on the genome sequence of *C. trachomatis* serovar D (http://chlamydia-www.berkeley.edu:4231/). The PCR fragment was gel purified and cloned into the *Eco*RI site of pBAD30. This plasmid was then digested with *Sac*I-*Sph*I and the liberated *murA* fragment was ligated into the *Sac*I-*Sph*I-digested pBAD18 to create pAJM6. Sequencing of the cloned *C. trachomatis*

Table 1.1. Bacterial strains and plasmids.

Strain or plasmid	Relevant Genotype	Source or Reference
Strain		
CodonPlus	*E. coli* BL21-CodonPlus-(DE3)-RIL [F^- *ompT hsdS*(r_B^- m_B^-) *dcm*$^+$ Tet^r *gal* λ (DE3) *endA* Hte (*argU ileY leuW* Cam^r)	Stratagene
ZK1746	*E. coli* W3110 *tna*2 Δ*lacU*169 Δ*murA::kan* (pBAD30-Z)	(Brown *et al.*, 1995)
ATM580	CodonPlus transformed with pAJM5	This work
ATM581	ATM580 Δ$murA_{Ec}$::*kan*	This work
ATM586	CodonPlus transformed with pAJM6	This work
ATM587	ATM586 Δ$murA_{Ct}$::*kan*	This work
C. trachomatis	*C. trachomatis* serovar L2	H. Caldwell
2457T	*S. flexneri* 2a wild type	(Formal *et al.*, 1958)
Plasmids		
pBAD30	Arabinose-inducible expression vector; Amp^r; p15A origin; low copy number	(Guzman *et al.*, 1995)
pBAD33	Arabinose-inducible expression vector; Cm^r; p15A origin; low copy number	(Guzman *et al.*, 1995)
pBAD18	Arabinose-inducible expression vector; Amp^r; pMB1 origin; low copy number	(Guzman *et al.*, 1995)
pBAD30-Z	pBAD30::$murA_{Ec}$; coding sequence and RBS[a] of *murA* from *E. coli* (W3110)	(Brown *et al.*, 1995)
pAJM5	pBAD18::$murA_{Ec}$; coding sequence and RBS of *murA* from *E. coli* (W3110)	This work
pAJM6	pBAD18::$murA_{Ct}$; coding sequence and RBS of *murA* from *C. trachomatis* serovar L2	This work

serovar L2 *murA* gene was performed by the Biomedical Instrumentation Center at USUHS. The sequence of the *C. trachomatis* serovar L2 *murA* has been deposited in GenBank under accession number AY152390.

Deletion of the chromosomal copy of *murA*. The strategy used to delete the essential *murA* gene from the chromosome of *E. coli* has previously been described (Brown *et al.*, 1995). BL21-CodonPlus-(DE3)-RI (CodonPlus) was first transformed with pAJM5 or pAJM6 and selected on medium containing ampicillin and chloramphenicol to generate ATM580 and ATM586. Next, a generalized transducing lysate of P1L4 grown on ZK1746 was used to transduce these strains to kanamycin resistance. Transductants were selected on LB agar containing ampicillin, chloramphenicol, kanamycin, and arabinose. Potential Δ*murA::kan* mutants were screened for arabinose-dependent growth by inoculating glucose-containing medium and observing growth after overnight (O/N) incubation at 37°C. Arabinose-dependent mutants were then screened for Δ*murA* and the presence of the *kan* gene by PCR. Primers A (5'-CACTACTGGCGGAAGAACCGG-3') and B (5'-ACAGAACGCAGTTGATGCGTAG-3') amplify a 750 bp fragment from an internal site of the *E. coli murA* gene to an internal site in the 5' upstream flanking gene (*yrbA*). Primers K (5'-CACCCCTTGTATTACTGTTTATGT-3') and B amplify a 550 bp fragment from a site internal to the *kan* gene to the *yrbA* gene. Because the *yrbA* gene is not present in pAJM5 or pAJM6, any PCR product obtained from primers A-B result from amplification of the genomic *murA*.

Growth and viability curves. Strains were grown O/N at 37°C with aeration. O/N cultures were diluted in 12.5 ml of LB containing appropriate antibiotics and arabinose (or glucose) in order to give an initial OD_{600} reading of 0.001. Cultures were

grown at 37°C with aeration and the OD_{600} of the samples was measured at various times to obtain the growth curve. For the viability curve, the same procedure was followed except that dilutions of the sample were plated on LB containing arabinose such that 30 to 300 colony forming units (cfu) were obtained per plate.

Phase contrast microscopy. Cells were grown to mid-exponential (OD_{600} = 0.6) and stationary phase (O/N cultures) at 37°C and viewed with an Olympus BX60 system microscope. All images were obtained with a SPOT RT CCC digital camera (Diagnostic Instrument, Inc.).

Assay of MurA activity. Three milliliters of O/N cultures of ATM581 and ATM587 were harvested and washed three times with ice cold 50 mM Tris, pH 7.5. Cells were resuspended in 1 ml of 50 mM Tris, pH 7.5, 2 mM DTT and sonicated three times for 15 second pulses using a sonication probe. Cell debris was removed from the samples by centrifugation for five minutes and the sample supernatant was desalted using a Pharmacia NAP-10 column equilibrated with 50 mM Tris, pH 7.5, 2 mM DTT. Protein concentration of the lysates was determined using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA).

The assay mixture (final volume 50 μl) contained 50 mM Tris of varying pH, 2 mM DTT, 10 mM UDP-GlcNAc and 10 μg of bacterial extract protein. Samples were pre-incubated for 15 minutes at 37°C and the reaction was started by the addition of 5 μl of 10 mM PEP. After 1 hour or 4 hours of incubation for ATM581 and ATM587 lysates, respectively, 800 μl of color reagent (1% ammonium molybdate, 1N HCl, 0.15% malachite green) was added to stop the reaction and measure the release of Pi. Results are expressed as OD_{660}, corrected for the background reading in the absence of UDP-GlcNAc. To

determine MurA activity in the presence of FOS, the pre-incubation samples contained FOS at concentrations ranging from 10 to 500 μg/ml .

Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of FOS. MIC assays for *C. trachomatis* serovar L2 and *Shigella flexneri* 2a were performed with varying concentrations of FOS in a plaque assay (Banks *et al.*, 1970; Oaks *et al.*, 1985). Confluent monolayers of L2 mouse fibroblast cells were infected for 90 minutes at 37°C, 5% CO_2 with 6 x 10^8 plaque forming units (pfu) of *C. trachomatis* serovar L2 or 6 x 10^6 cfu of *S. flexneri* 2a. After 90 minutes the infection medium was removed. *C. trachomatis* and *S. flexneri*-infected cells were incubated for 14 and 3 days, respectively, at 37°C, 5% CO_2 after addition of a 5 ml agarose overlay (0.75% low melting point agarose) containing Dulbecco's Modified Eagles Medium (DMEM) , fetal bovine serum (FBS) (10%), gentamicin (20 μg/ml), cyclohexamide (20 μg/ml), and varying concentrations of FOS. For *C. trachomatis*-infected cells, a second 5 ml agarose overlay was added at day 7. In order to visualize plaques at the end of the incubation period, cells were stained with 0.5% neutral red for 2 hours at 37°C, 5% CO_2.

In order to determine the MBC of FOS for CodonPlus, ATM581 and ATM587, strains were grown O/N at 37°C with aeration and diluted 1:100 into fresh medium containing arabinose. Cultures were grown to mid-exponential phase and adjusted to an OD_{600} of 0.5 in order to normalize all cultures to the same initial OD. One-hundred microliters of dilutions of each culture were plated on LB agar containing arabinose and varying concentrations of FOS.

RT-PCR. L2 mouse fibroblast cells were infected with *C. trachomatis* serovar L2 at an MOI of 1 for two hours at 37°C, 5% CO_2. After the infection period, the infection

medium was replaced with medium containing DMEM, FBS (10%), and gentamicin (20 μg/ml). At various times post-infection, the cells were removed and total RNA from each sample was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's specifications. Total RNA was reverse transcribed using the Thermoscript reverse transcription system (Invitrogen, Carlsbad, CA) and the resulting cDNA was screened for *murA* and *murB* transcripts by PCR using Taq polymerase. Primers OAM2 (5'CTTGTAGCCTCCTTACTTTCGGA) and OAM3 (5' GTGATGTGTGCTCCAACAAGA) were expected to generate a 400 bp fragment from *C. trachomatis murA* mRNA. Primers OAM10 (5' CGGGATTAGAATTTGCCGTA) and OAM11 (5' ATATCTGCCCGCCTCCTATC) were expected to generate a 400 bp fragment from *C. trachomatis murB* mRNA.

RESULTS

Cloning of *murA* from *C. trachomatis.* In order to determine whether the chlamydial *murA* encoded a functional enzyme, we cloned the gene from *C. trachomatis* serovar L2. The –10 and complete coding region of *murA* from *C. trachomatis* serovar L2 was PCR amplified using primers designed from the published genome sequence of *C. trachomatis* serovar D (Stephens *et al.*, 1998). The resulting PCR product was cloned into pBAD18 to place the gene under the arabinose-inducible, glucose-repressible *ara* promoter. The resulting clone, pAJM6, was sequenced a minimum of four times on each strand to confirm that the insert contained the *C. trachomatis murA.* Amino acid alignment of the MurA of *C. trachomatis* serovar D and *C. pneumoniae* against our *C. trachomatis* serovar L2 sequence revealed 99 and 66 % similarity, respectively. The sequence of *E. coli* MurA was 32% similar to MurA of *C. trachomatis* serovar L2.

Complementation of *E. coli* Δ*murA* by *murA*$_{ct}$. Previous studies in *E. coli* have revealed that a single copy of *murA* exists in the genome. By cloning the wild-type *E. coli murA* under the control of the arabinose-inducible, glucose-repressible *ara* promoter, it was shown that loss of *murA* from the *E. coli* chromosome is lethal as the *ΔmurA* mutants are only viable in the presence of arabinose (*i.e.* when the cloned *murA* is expressed *in trans*) (Brown *et al.*, 1995). To determine if the chlamydial *murA* could complement a lethal *murA* deletion in *E. coli*, CodonPlus was transformed with pAJM6 (pBAD18::*murA*$_{ct}$) and then a *ΔmurA::kan* mutation was introduced by transduction to kanamycin resistance. Transductants arose at a frequency of 1.8×10^{-7}. One transductant was chosen as ATM587 (ctMurA). As a control *E. coli* CodonPlus was transformed with pAJM5 (pBAD18::*murA*$_{ec}$) and transduced to kanamycin resistance as above. Transductants arose

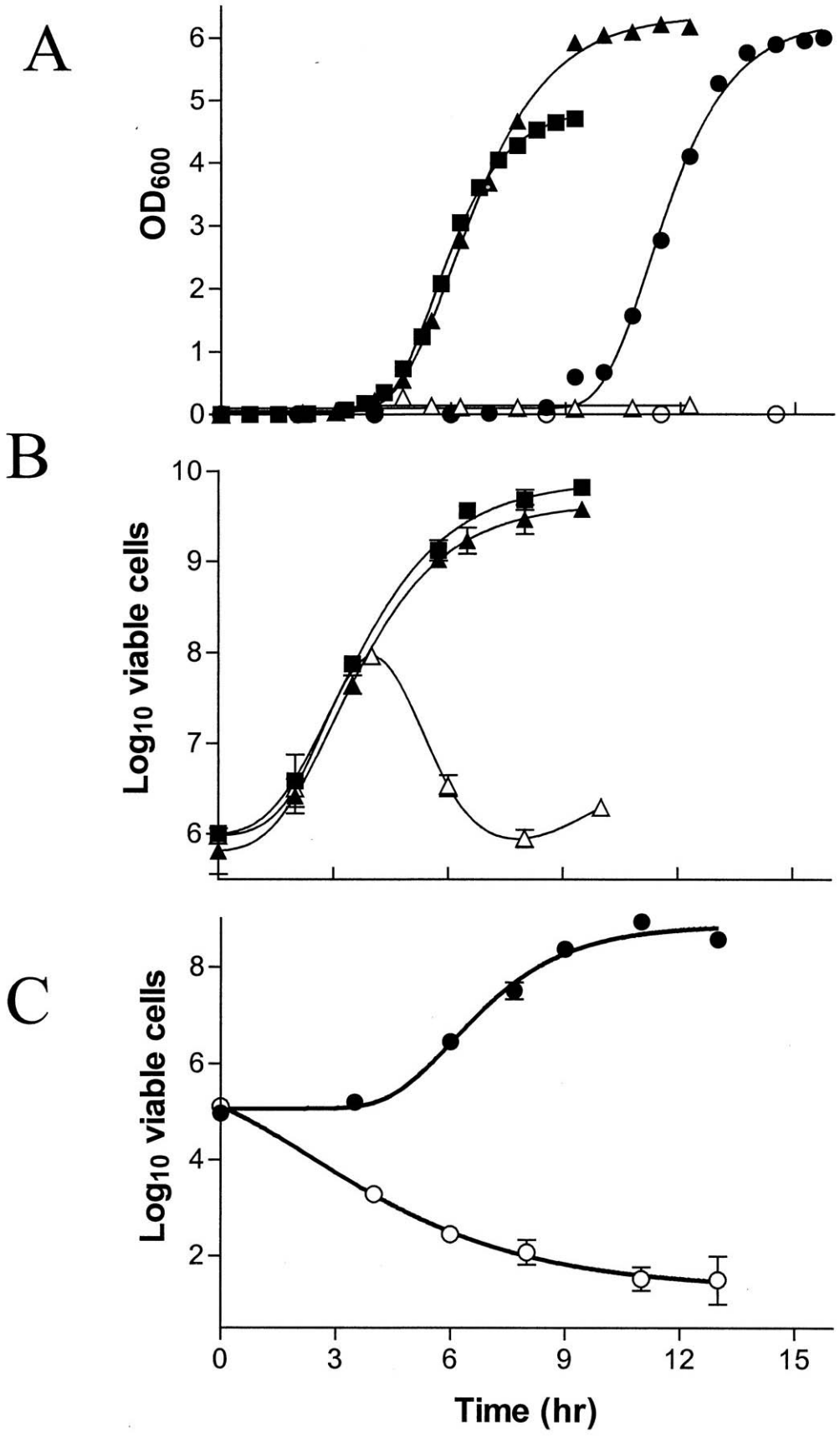
at a frequency of 7.1 x 10^{-7}. One transductant was chosen as ATM581 (ecMurA). After 18 hours growth on LB arabinose medium at 37°C, a marked difference in colony size was observed between ATM581 and ATM587 with the strain expressing $murA_{ct}$ being smaller. To test growth dependence on the expression of the cloned *murA*, ecMurA and ctMurA were inoculated into LB containing 0.2% glucose or 0.1% arabinose. For both strains growth occurred only when *murA* expression was induced with arabinose although there was some leaky expression of the *E. coli murA*.

We confirmed deletion of *murA* in ATM581 and ATM587 by PCR (data not shown). In order to distinguish chromosomal *murA* from plasmid *murA*, we chose a primer that anneals upstream of *murA* to the *yrbA* gene and a primer that anneals to *murA*. Plasmid encoded *murA* does not contain sequence from *yrbA* and therefore will not produce false positive PCR data. Only the *E. coli* CodonPlus control generated a PCR fragment using *yrbA* and *murA* primers. In contrast, using primers that anneal to *yrbA* and *kan*, ATM581 and ATM587 but not the *E. coli* CodonPlus control generated PCR fragments.

In the presence of arabinose, the OD_{600} of overnight cultures of ctMurA was significantly lower than that of ecMurA (data not shown). This observation as well as the fact that ctMurA grew slower than ecMurA on agar supplemented with arabinose, suggested that while chlamydial *murA* complemented the *ΔmurA* of *E. coli*, it affected the kinetics of growth differently than the cloned *E. coli murA* gene.

Growth and viability curves. The growth rate of ecMurA and ctMurA was measured by OD and compared to the parent strain ($murA^+$). Figure 1.2A depicts the time course of growth for each strain in the presence of arabinose or glucose. As expected, under

Figure 1.2. Growth and viability of ecMurA and ctMurA under arabinose-inducing and glucose-repressing conditions. *E. coli* CodonPlus (squares), ecMurA (triangles) and ctMurA (circles) were inoculated into medium containing arabinose (filled symbols) or glucose (open symbols). At various times the OD_{600} (A) was measured in order to monitor the growth of the cultures or dilutions (B and C) were plated onto LB media containing arabinose to assess the viability of the cultures. Viability platings were performed in triplicate.


A
B
C
OD600
Log10 viable cells
Log10 viable cells
Time (hr)
0
1
2
3
4
5
6
7
8
9
10
0
3
6
9
12
15

glucose-repressing conditions neither Δ*murA* mutant was able to grow. When the cloned *murA* was induced by the addition of arabinose to the culture, the growth of ecMurA followed the same rate as wild type *E. coli* CodonPlus over time. ctMurA also exhibited a similar growth rate, but a much longer lag phase occurred before the culture entered the logarithmic stage. This lag phase could be the result of the chlamydial MurA being less active than the *E. coli* MurA, a poor RBS recognition in the chlamydial coding sequence, poor expression due to codon usage difference in *E. coli* or fewer viable cells in the overnight culture of the *E. coli* strain expressing the chlamydial MurA. When grown with arabinose, both complemented mutants reached a similar plateau, which was a higher OD_{600} value than that of the wild-type parent. This result suggested that either more cells were present or the cell morphology had been altered.

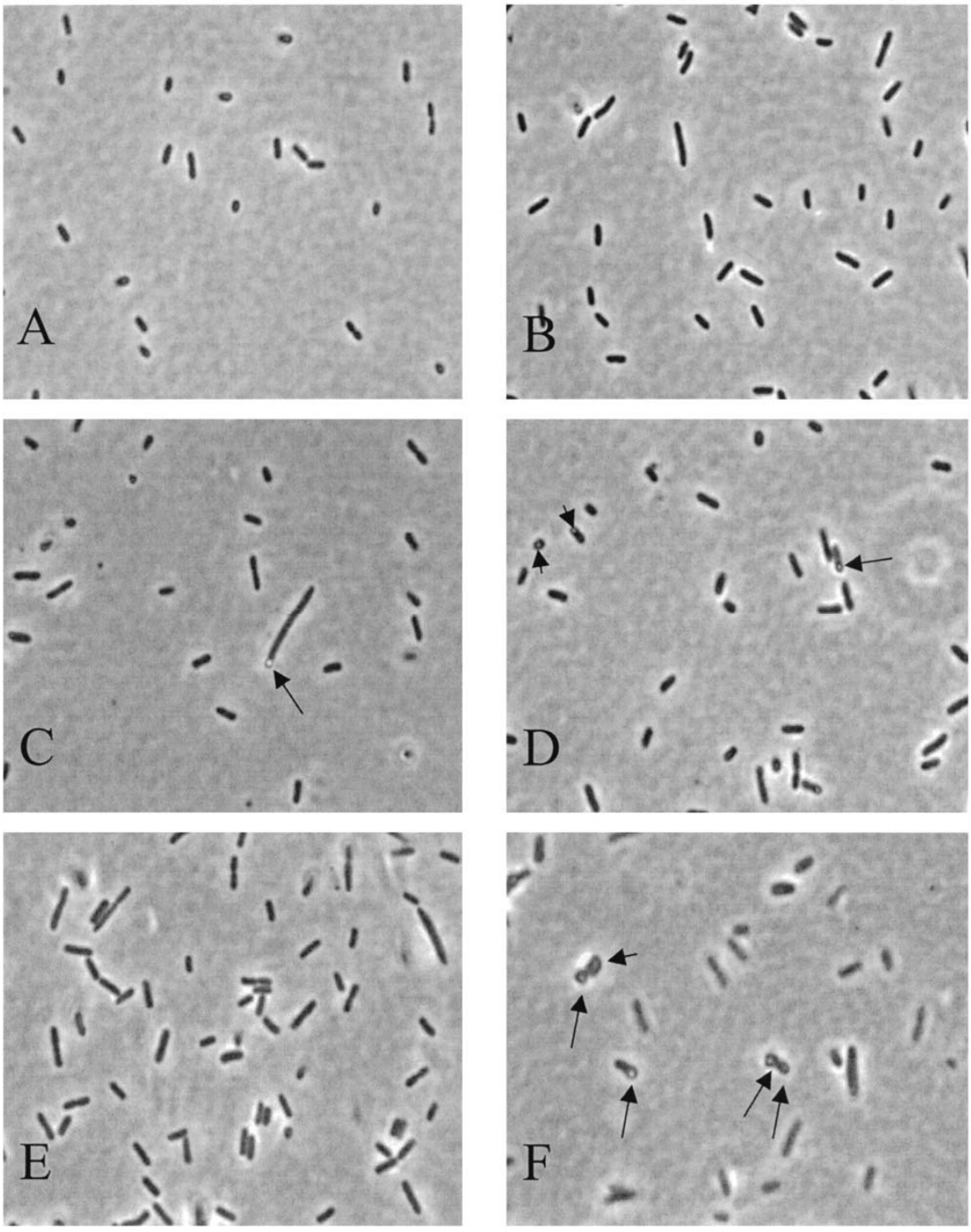
In order to determine the viable numbers of ctMurA and ecMurA under inducing and repressing conditions, bacteria were grown in LB containing arabinose or glucose and plated on agar containing arabinose. In the presence of arabinose or glucose the number of viable cells of ecMurA was the same as that for the wild-type parent during the first four hours of growth (Figure 1.2B). After four hours however, ecMurA grown in glucose-containing media quickly lost viability. The initial increase in bacterial numbers of ecMurA under glucose-repressing conditions was probably due to residual activity of MurA remaining in the cells from overnight growth with arabinose. Viability decreased as turn-over of MurA occurred because no new MurA was being made under the repressing conditions. The number of viable ecMurA cells in the presence of arabinose paralleled the number of viable *E. coli* CodonPlus cells throughout the course of the experiment (Figure 1.2B). Consistent with the OD_{600} readings in Figure 1.2A, the viability of ctMurA in the

presence of arabinose was similar to *E. coli* CodonPlus and ecMurA except for an initial lag period (Figure 1.2C). However, the initial and final viable counts of ctMurA were each one order of magnitude lower than those of *E. coli* CodonPlus and ecMurA even though the OD_{600} of each culture was the same at time zero. In contrast to what was observed for ecMurA in the presence of glucose, no initial growth of ctMurA occurred and viability of the culture gradually diminished to approximately 100-1000 cfu/ml after 10 hours.

Cellular morphology. Cell morphology was examined using phase contrast microscopy. Compared to the uniformly small rod shape of *E. coli* CodonPlus from O/N cultures (Figure 1.3A), both complemented mutants appeared longer and more filamentous (Figure 1.3B-D). One striking characteristic of approximately 25% of the ctMurA cells was the presence of bulbs polarized at one end of the bacterium (Figure 1.3C-D). Similar bulbs have been observed in cell wall mutants of *E. coli* (Denome *et al.*, 1999; Karow *et al.*, 1991; Schwarz *et al.*, 1969; Spratt, 1975). During logarithmic growth, these bulbs were observed as transparent holes in the center of both daughter cells as they were dividing (Figure 1.3F). Similar bulbs were not seen in logarithmic cultures of ecMurA (Figure 1.3E).

***In vitro* activity of *Chlamydia* MurA.** The addition of enolpyruvate to UDP-GlcNAc by MurA releases orthophosphate (Pi). This phosphate release can be measured optically and used to indirectly assess the activity of MurA. DNA sequence analysis predicted that the *C. trachomatis* serovar L2 MurA contains a cysteine to aspartate (C→D) change at amino acid 119 in the active site of the enzyme. Kim *et al* previously showed that an *E. coli* MurA mutant containing the same C→D substitution in the active site alters the pH optimum for the enzyme. The activity of the wild-type *E. coli* MurA remains

Figure 1.3. Morphology of ecMurA and ctMurA cells. Overnight cultures of *E. coli* CodonPlus (A), ecMurA (B) and ctMurA (C and D), and logarithmic phase cultures of ecMurA (E) and ctMurA (F) were visualized by phase contrast microscopy. Black arrows point to the presence of bulbs in approximately 25% of the ctMurA cells.


A
B
C
D
E
F

relatively constant over a pH range of 5 to 9. In contrast, the mutant MurA has greater activity than the wild-type MurA at pH values less than 7 and dramatically loses activity at pH values greater than 7 (Kim *et al.*, 1996). To test whether the activity of the *Chlamydia* MurA was also pH sensitive, the release of Pi from a crude lysate of ctMurA was measured over a pH range of 5.5 to 8.0. Because measurement of Pi release required saturation kinetics, we determined that for ecMurA saturation was reached by one hour, whereas it took four hours to reach saturation for ctMurA (data not shown). Figure 1.4 represents the rate of Pi release by chlamydial MurA at saturating concentrations of substrate at different pH values. As predicted from the *E. coli* mutant data, Pi release was maximal at pH values less than 7.0. Further *in vitro* characterization of the chlamydial MurA activity was performed at pH 6.6.

Previous studies have also demonstrated that the C→D conversion in the MurA active site renders the enzyme resistant to FOS (De Smet *et al.*, 1999; Kim *et al.*, 1996). In order to determine if the same phenotype was true for the chlamydial MurA, crude lysates of ecMurA and ctMurA were tested for their ability to release Pi in the presence of varying concentrations of FOS. As expected, wild type *E. coli* MurA activity was nearly abolished in the presence of concentrations of FOS as low as 0.5 μg/ml (Figure 1.5A) and no further reduction in activity was seen at concentrations up to 10 μg/ml. Incubation of ecMurA for four hours in the presence of FOS did not increase the amount of Pi release from that of one hour incubation (data not shown), suggesting that the *E. coli* enzyme is completely inactivated at these concentrations of FOS. Unlike ecMurA, ctMurA was strikingly resistant to FOS and MurA activity was detected in the presence of concentrations of FOS up to 500 μg/ml (Figure 1.5B). Taken together, these data suggest that the chlamydial

Figure 1.4. pH dependence of *C. trachomatis* serovar L2 MurA activity in vitro.

Whole cell lysates of ctMurA were preincubated with 10 mM UDP-GlcNAc, 2 mM DTT, and 50 mM Tris at the pH indicated. 10 mM PEP was added to each sample to start the reaction. After 4 hours incubation, release of inorganic phosphate was measured by adding color reagent and measuring the OD_{660} of the sample (described in Materials and Methods). Inorganic phosphate release was measured in triplicate.

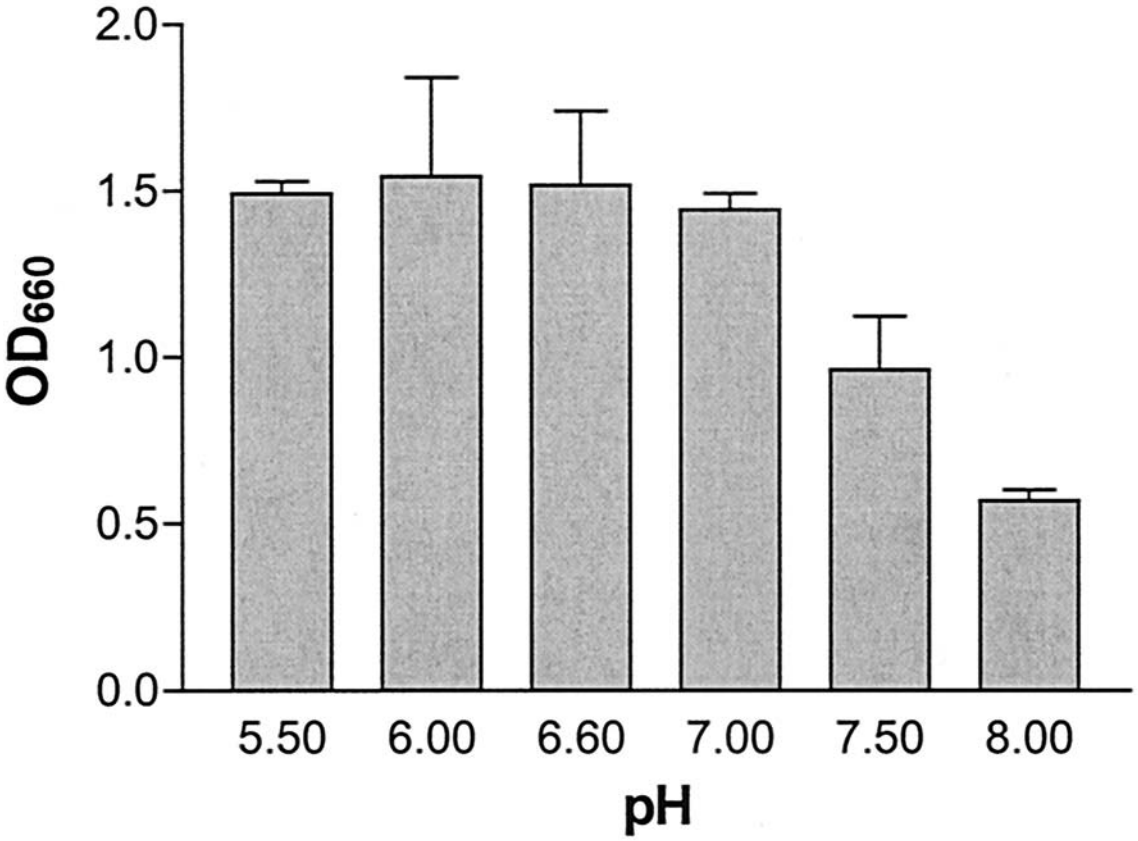
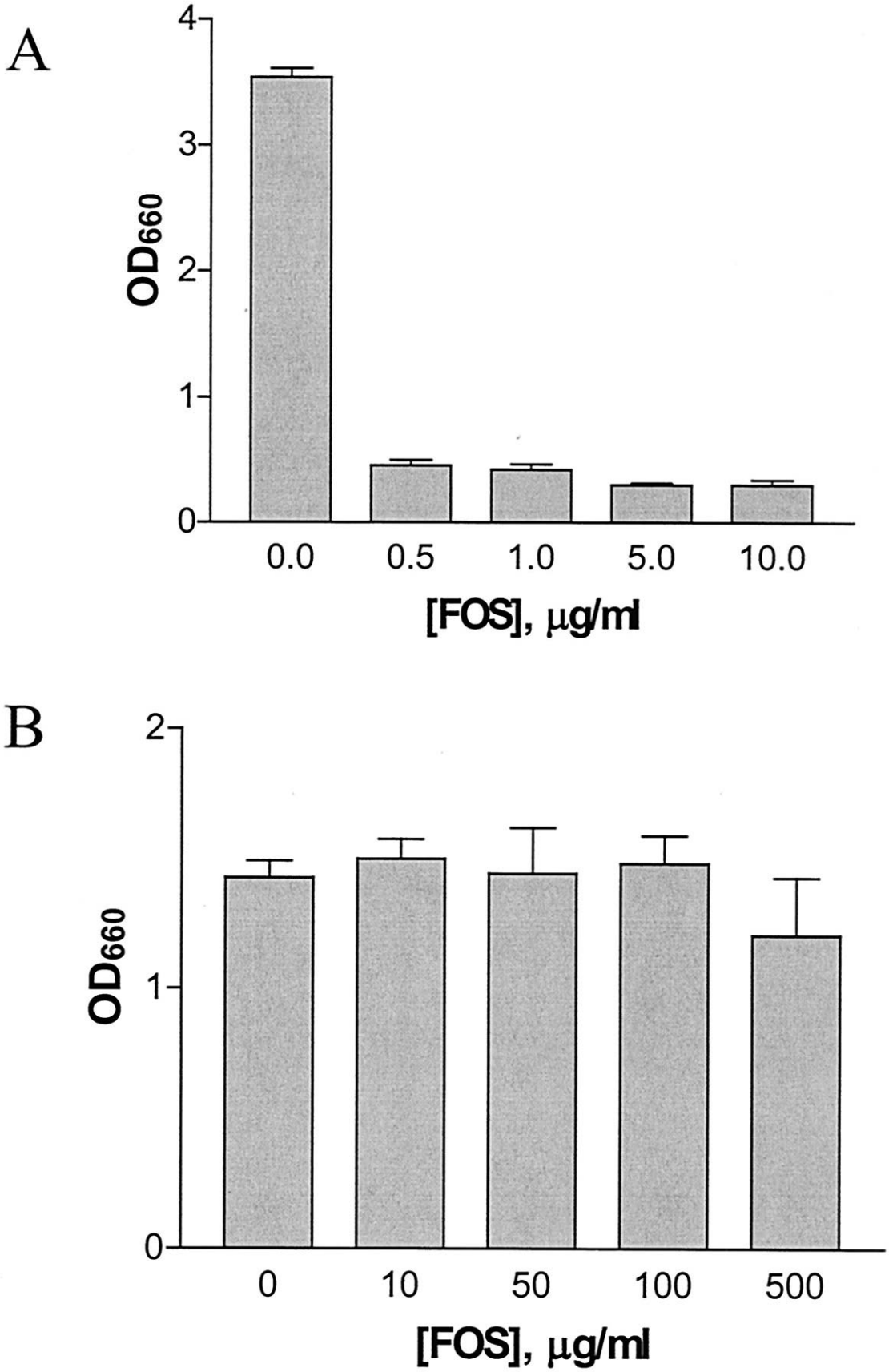

2.0
1.5
1.0
0.5
0.0
OD660
5.50
6.00
6.60
7.00
7.50
8.00
pH

Figure 1.5. *In vitro* MurA activity in the presence of fosfomycin. Whole cell lysates of ecMurA (A) and ctMurA (B) were preincubated with 10 mM UDP-GlcNAc, 2 mM DTT, indicated fosfomycin concentrations, and 50 mM Tris, pH 7.5 (A) or pH 6.6 (B). 10 mM PEP was added to start the reaction. After 1 hour incubation (A) and 4 hours (B) incubation, release of inorganic phosphate was measured by adding color reagent and determining the OD_{660} of the sample. Inorganic phosphate release was measured in triplicate.


A
4
3
2
1
0
OD660
0.0
0.5
1.0
5.0
10.0
[FOS], µg/ml
B
2
1
0
OD660
0
10
50
100
500
[FOS], µg/ml

MurA is a functional enzyme that can catalyze the addition of enolpyruvate to UDP-GlcNAc and is resistant to FOS.

***In vivo* resistance of *C. trachomatis* MurA to FOS.** *Chlamydiae* are obligate intracellular eubacteria. Because chlamydiae require host eukaryotic cells for growth, MIC assays cannot be performed in broth or on L-agar. To determine whether resistance of ctMurA to FOS *in vitro* correlated to resistance *in vivo*, we examined growth of *Chlamydia* in a plaque assay (Banks *et al.*, 1970). In this assay, *Chlamydia* is allowed to infect confluent monolayers of mouse fibroblast cells. After a 120 minute infection period, the infection medium is replaced by an agarose overlay. This overlay contains nutrients to support the viability of the eukaryotic cells. When newly replicated EBs are released from infected cells, the agarose overlay keeps the released EBs localized such that they can only re-infect neighboring cells. As infection, release, and re-infection of the eukaryotic cells by *Chlamydia* proceeds, a plaque is formed in the monolayer of eukaryotic cells that can be observed after the cells are stained with neutral red. Each plaque arises from initial infection of a single eukaryotic cell. Effectiveness of any inhibitor of chlamydial growth can be assessed by addition of the compound to the agarose overlay and comparison of the number of plaques that form in the presence and absence of the compound.

In our laboratory, we also use the plaque assay to determine the effects of compounds on the intracellular growth of *Shigella flexneri*, a facultative intracellular bacterial pathogen closely related to *E. coli*. Like *Chlamydia*, *S. flexneri* infects mouse fibroblast cells and spreads to neighboring cells, forming plaques in the monolayer (Oaks *et al.*, 1985). However, *Shigella* is sensitive to FOS when grown on L-agar. Therefore, as a control for drug penetration into fibroblast cells, plaque formation by *S. flexneri* 2a was

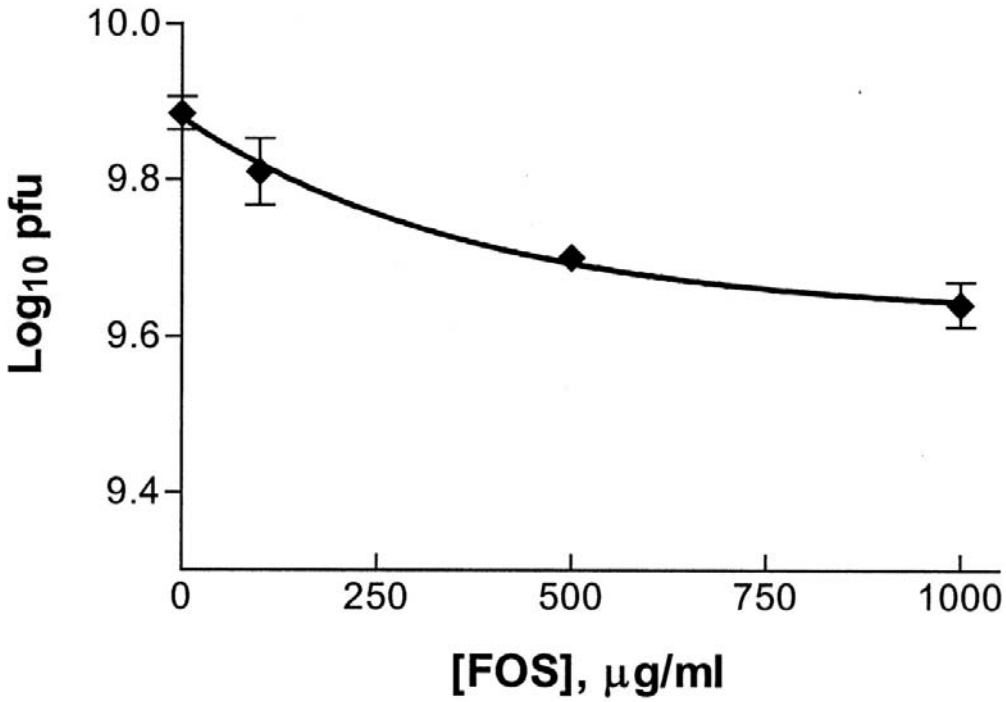
determined in the presence and absence of FOS. At a FOS concentration of 1 μg/ml, plaque formation by *S. flexneri* 2a was completely abolished (data not shown) suggesting that the drug is able to be efficiently taken up by eukaryotic cells and can inhibit plaque formation by FOS-sensitive organisms.

Next, we tested the effect of FOS on *C. trachomatis* serovar L2 in the plaque assay. As seen in Figure 1.6A, concentrations of FOS up to 1000 μg/ml did not significantly inhibit plaque formation of *C. trachomatis* in the plaque assay. Although no dose-dependent inhibition in the ability of *C. trachomatis* serovar L2 to form plaques was observed, a dose-dependent decrease in the average plaque size was observed at concentrations up to 500 μg/ml (Figure 1.6B). At higher concentrations of FOS the average plaque size returned to sizes similar to that seen in the absence of FOS. It is not understood why the plaque sizes increased at higher concentrations of the antibiotic. It is unlikely that toxic effects of FOS on the fibroblast cells account for the increase in plaque size as uninfected, FOS-treated controls displayed normal morphology in this assay. In this same assay, *C. trachomatis* serovar D and *C. psittaci* Cal10 and GPIC were also resistant to FOS and formed plaques at concentrations up to 500 μg/ml of FOS (data not shown).

Because the in vitro and in vivo data suggested that the chlamydial MurA was responsible for the resistance of *Chlamydia* to FOS, we wanted to verify this resistance by asking whether the chlamydial MurA could impart resistance to the sensitive *E. coli* strain. Therefore, the MBC of FOS was determined for *E. coli* CodonPlus, ecMurA and ctMurA. Figure 1.7A represents the MBC of FOS for *E. coli* CodonPlus. A dramatic decrease in viability was observed at a concentration as low as 5 μg/ml and growth of this strain was completely inhibited at 200 μg/ml of FOS. When the *Chlamydia* murA was expressed *in*

Figure 1.6. MIC of fosfomycin for *C. trachomatis* serovar L2. Confluent mouse fibroblast monolayers were infected with *C. trachomatis* serovar L2 as described in Materials and Methods. At 2 hours post-infection, a 0.5% agarose overlay containing indicated concentrations of fosfomycin was added. (A) The $\log_{10}$ of the plaque forming units/ml was calculated after 14 days. (B) Plaque size was measured for 10 individual plaques and the average was plotted against the fosfomycin concentration.

A


10.0
9.8
9.6
9.4
Log10 pfu
0
250
500
750
1000
[FOS], μg/ml

B

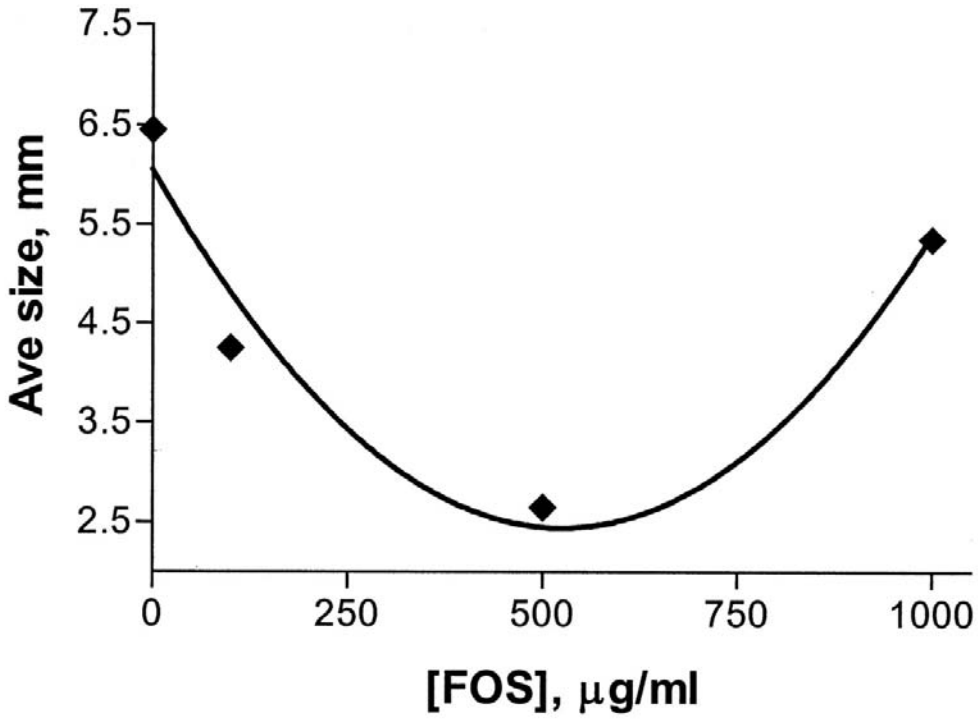
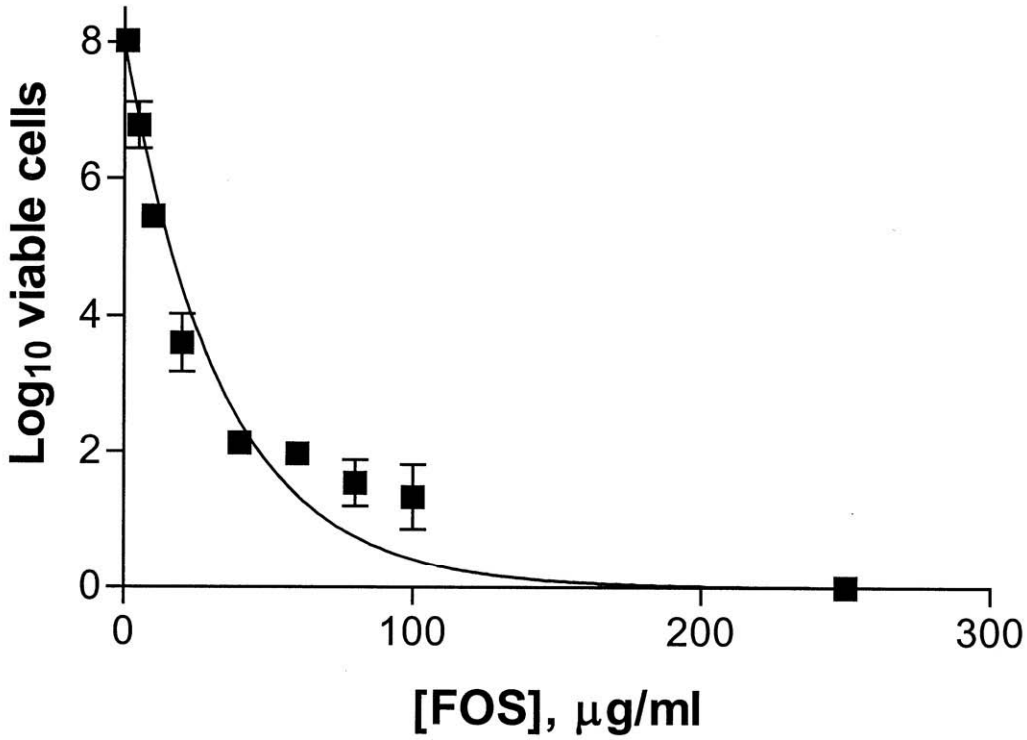

7.5
6.5
5.5
4.5
3.5
2.5
Ave size, mm
0
250
500
750
1000
[FOS], μg/ml

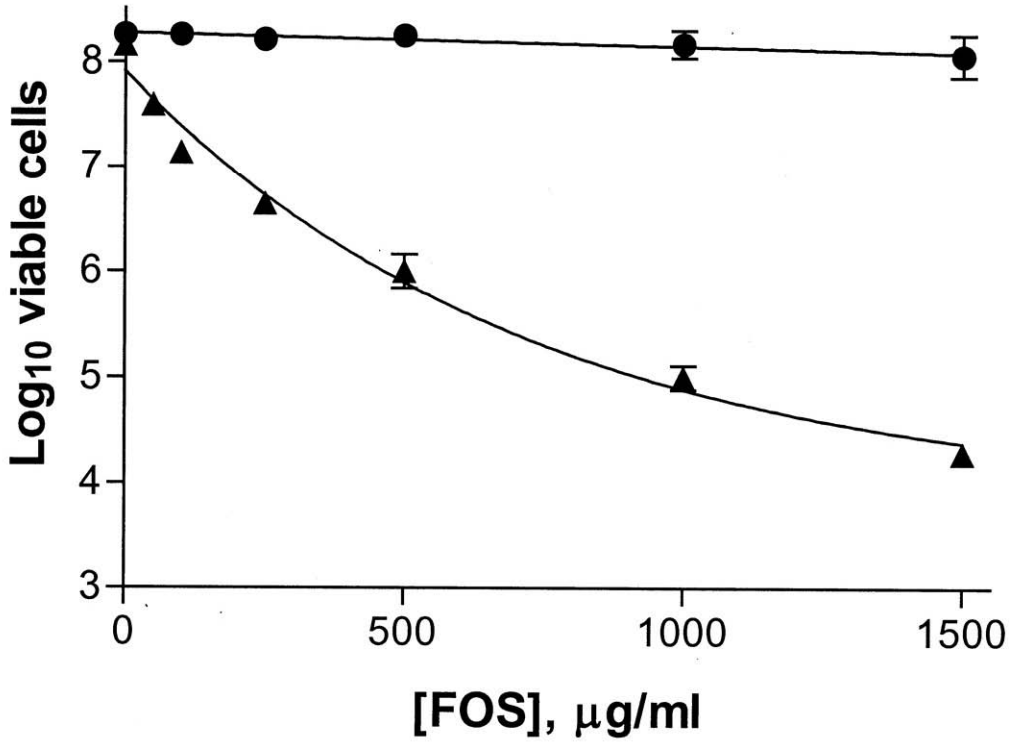
Figure 1.7. MBC of fosfomycin for *E. coli* CodonPlus, ecMurA and ctMurA.

Logarithmic phase cultures were diluted in LB and plated on LB agar containing indicated fosfomycin concentrations. The $\log_{10}$ of the viable cells/ml was calculated for (A) *E. coli* CodonPlus, and (B) ecMurA (triangles) and ctMurA (circles) and plotted against fosfomycin concentration.

A


Log10 viable cells
8
6
4
2
0
0
100
200
300
[FOS], μg/ml

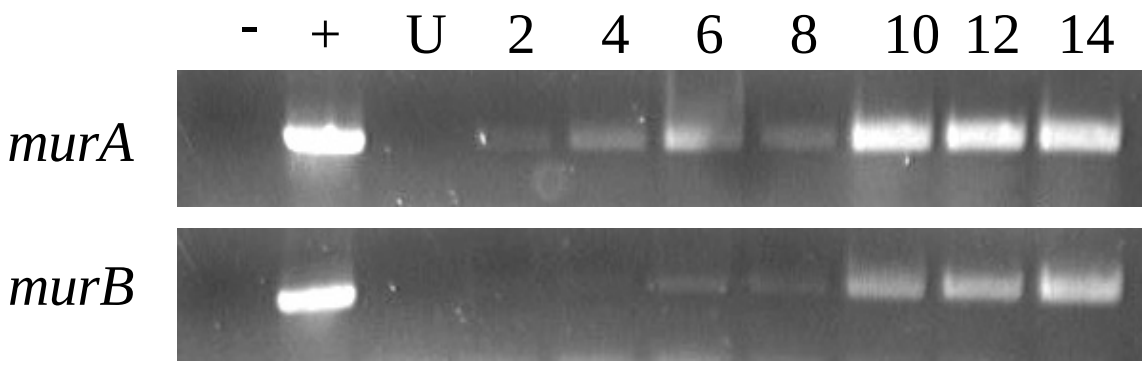
B


Log10 viable cells
8
7
6
5
4
3
0
500
1000
1500
[FOS], μg/ml

trans (ctMurA), the strain displayed complete resistance to FOS at concentrations up to 1500 μg/ml (Figure 1.7B). This resistance was due to enzyme activity of *Chlamydia* MurA and not because of high level expression of $murA_{ct}$ from a low copy number vector. Some resistance to FOS was also observed for ecMurA ($murA_{ec}$ cloned on the same low copy number plasmid as $murA_{ct}$) but this strain exhibited increased sensitivity as the FOS concentration increased (Figure 1.7B). The difference in FOS resistance between ecMurA and ctMurA was four orders of magnitude at 1500 μg/ml. These data suggest that C. trachomatis serovar L2 resistance to the cell wall inhibitor FOS is due to synthesis of a resistant form of MurA.

RT-PCR of *murA* from *C. trachomatis* serovar L2. The genome sequences of *C. trachomatis* and *C. pneumoniae* contain homologues of genes in the PG biosynthesis pathway, including *murA*. While we could demonstrate *in vitro* activity of *Chlamydia* MurA and *in vivo* activity in *E. coli*, we wanted to determine if *murA* mRNA was expressed in *Chlamydia* at any time during the chlamydial life cycle. L2 mouse fibroblast cells were infected with *C. trachomatis* serovar L2 and total RNA was extracted from *Chlamydia*-infected cells at various times post-infection. Figure 1.8 depicts the time course of expression for *C. trachomatis murA* mRNA. *murA* mRNA transcripts were detected as early as 2 hpi and expressed throughout the timecourse of the chlamydial infection. This expression profile was similar to that of *C. trachomatis hsp60* (data not shown and (Shaw *et al.*, 2002). We also examined the expression of *C. trachomatis murB* mRNA. *murB* encodes a UDP-*N*-GlcNAc enolpyruvyl reductase, which reduces UDP-GlcNAc enolpyruvate to UDP-MurNAc (Figure 1.1). MurNAc is the hallmark constituent of PG.

Figure 1.8. Detection of *C. trachomatis* serovar L2 gene transcripts by RT-PCR during infection. RT-PCR analysis was performed for *murA* and *murB* mRNA isolated from infected cells at the indicated time points after infection. "-": RNA/DNA negative control; "+": genomic DNA positive control; "U": uninfected cells.


- + U 2 4 6 8 10 12 14
murA
murB

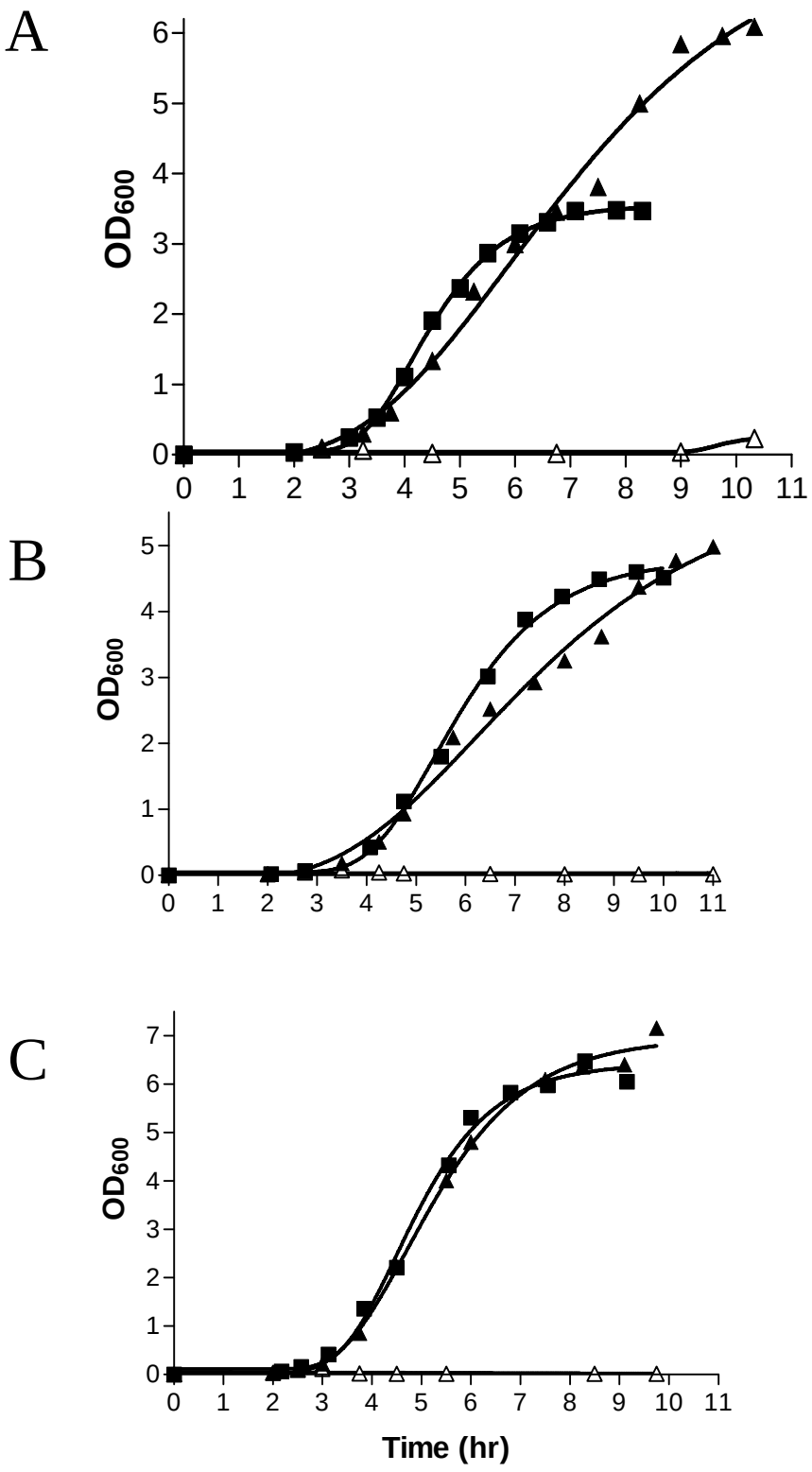
We were able to detect *murA* mRNA transcripts, however expression lagged behind the expression of *murA* mRNA (Figure 1.8).

DISCUSSION

Despite the lack of evidence for PG in *Chlamydia*, a nearly complete *E. coli* PG synthesis pathway is encoded in the genomes of each *Chlamydia* spp (Figure 1.1). Until now, the functional assessment of an enzyme in the PG biosynthesis pathway in *Chlamydia* has not been performed. Here, we have demonstrated that the chlamydial MurA functions as an UDP-*N*-acetylglucosamine enolpyruvyl transferase and commits UDP-GlcNAc to PG synthesis. Using the strategy of Brown *et al* (4), we demonstrated the activity of the chlamydial MurA by its ability to restore viability to an *E. coli* Δ*murA* mutant. Growth and viability of the *E. coli* mutant was dependent on the arabinose-induced expression of the cloned chlamydial *murA* (Figure 1.2A and B).

Complementation of a *murA* mutation in *E. coli* demonstrated that the chlamydial MurA is capable of catalyzing the addition of enolpyruvate to UDP-GlcNAc. However complementation was strain dependent. The parent strain of all Δ*murA* mutants in this body of work is *E. coli* BL21 Codon Plus, an *E. coli* B strain. We also attempted to construct a Δ*murA E. coli* mutant expressing the *E. coli* and chlamydial *murA* in the *E. coli* K-12 strains, MC4100, C600 and χ2842. We were successful in constructing Δ*murA E. coli* mutants that expressed the *E. coli murA in trans* in all the *E. coli* K-12 backgrounds. The growth of these Δ*murA* mutants was dependent upon the expression of the *E. coli murA* from the *ara* promoter (Figure 1.9). Interestingly, altered growth kinetics, as assessed by measuring the OD_{600} over time and comparing to the wild-type parent, were observed in the *E. coli* MC4100 and C600 mutants when the *E. coli murA* was expressed *in trans*. However, the growth kinetics of χ2842 mirrored that of the wild-type parent and were similar to that the complementing *E. coli murA* in the *E. coli* B strain (Figure 1.2A).

Figure 1.9. Expression of $murA_{Ec}$ in various *E. coli* K-12 strains. Wild type (squares) and Δ*murA* mutants (triangles) were inoculated into medium containing arabinose. At various times the OD_{600} was measured in order to monitor the growth of the cultures. (A) MC4100, (B) C600, and (C) χ2842.


A
B
C
OD600
Time (hr)

When we tried to move the Δ*murA* mutation into the MC4100 background expressing the chlamydial *murA*, we were never successful in obtaining Kan^R transductants. On the other hand, we were able to obtain Kan^R transductants in both the C600 and χ2842 *E. coli* backgrounds that were expressing the chlamydial *murA.* However, these mutants displayed no growth dependence for the arabinose inducer (data not shown). Using PCR, we further determined that these mutants were positive for the presence of the chlamydial *murA* in the vector as well as both the *kan* cassette and *murA* gene in the genome suggesting that a gene duplication had occurred during the transduction (data not shown). One possible explanation for this host strain dependence is a difference in codon-usage in *Chlamydia* compared to *E. coli*. However, this does not seem to be the case as complementation of the Δ*murA* was achieved in a *E. coli* CodonPlus strain that had lost the plasmid encoding the extra tRNAs (data not shown). More likely, the *E. coli* CodonPlus strain carries a different genetic background than the other strains, which allows the chlamydial gene to be more efficiently expressed.

The altered morphology observed in ctMurA cells is quite interesting. Approximately 25% of the cells formed bulbs. These bulbs were polarized when the bacterial cultures were in stationary phase yet were found in the center of both daughter cells as they were dividing. It is unclear what caused these bulbs or why they only occurred in 25% of the population. A similar phenotype has been reported in other cell wall mutants of *E. coli*, including *htrB, pbpA and pbpB* mutants (Denome *et al.*, 1999; Karow *et al.*, 1991; Schwarz *et al.*, 1969; Spratt, 1975). HtrB is a lauroyl transferase, a late functioning acyltransferase of lipid A biosynthesis, and is required for growth above 32°C. The presence of bulbs in *htrB* mutants depends on the initial OD of the culture when the

culture is shifted to higher temperatures. At OD_{600} values below 0.05, the cells form bulbs and bulges, but when the cells are shifted to 42°C at higher OD_{600} values, the phenotype shifts from bulbs and bulges in the cells to a filamentous phenotype (Karow *et al.*, 1991). We have not explored whether the 25% of ctMurA cells would shift to a filamentous phenotype if the OD of the cultures was higher than 0.05. Regardless of the initial OD of the *htrB* mutant cultures, the cells eventually ceased dividing and lost viability (Karow *et al.*, 1991). It is possible that the 25% of ctMurA cells also cease dividing and this may explain the order of magnitude difference in total viable cells between ctMurA and ecMurA cultures.

In *E. coli* PBP2 and PBP3 are classified as high molecular mass penicillin-binding proteins and are essential to *E. coli* development (Goffin and Ghuysen, 1998). PBP2 and PBP3, encoded by *pbpA* (*mrdA*) and *pbpB* (*ftsI*), respectively, are transpeptidases involved in establishing the cell shape and cell division. PBP2 maintains the cell's rod shape. This has been demonstrated by the formation of round cells in *pbpA* (Ts) mutants and in *E. coli* treated with mecillinam, a β-lactam antibiotic that specifically targets PBP2 (Begg and Donachie, 1985; Spratt, 1975). PBP3 is involved in the formation of septal PG and is essential for cell division. In *pbpB* (Ts) mutants and in *E. coli* cultures treated with cephalexin, a β-lactam antibiotic that specifically targets PBP3, cell division ceases and filamentation occurs (Begg and Donachie, 1985; Spratt, 1975). Bulbs and bulges have not been identified in *Chlamydia* treated with β-lactam antibiotics, although *Chlamydia* genomes encode homologues of PBP2 and PBP3. However, abnormalities in morphology and cell division are observed. It should be noted that chlamydial RBs are not rod shaped bacteria but rather, they are round. Recently, it was shown that mecillinam specifically

targets the chlamydial PBP2 homologue and inhibits chlamydial cell division although the effect of mecillinam on cell morphology was not studied (Storey and Chopra, 2001). Perhaps, the role of PBP2 and PBP3 in the formation of chlamydial PG differs from their roles in *E. coli*.

We found that ctMurA displayed altered growth kinetics compared to the wild-type parent (*murA*$^{+}$) and ecMurA. A 5-6 hour lag occurred before ctMurA cultures entered the exponential phase of growth (Figure 1.2A). One possible explanation for this lag is the viability difference observed in ctMurA cultures (Figure 1.2B and C). Although the initial OD_{600} values of the ctMurA, ecMurA and wild type *E. coli* CodonPlus cultures were the same, the viability of ctMurA was one order of magnitude lower than ecMurA and wild type *E. coli* CodonPlus. Nevertheless, this explanation cannot account for the lag in time before the cells began to divide (Figure 1.2C). A difference in the activity of the chlamydial MurA is more likely to be responsible for the altered growth and viability kinetics. The optimal enzyme activity of the chlamydial MurA is at pH values less than 7 and a significant decrease in activity is seen at pH 7.5. In *E. coli*, the cytoplasmic pH is maintained at 7.5 over an extracellular pH range of 5 to 9 (Padan *et al.*, 1981; Slonczewski *et al.*, 1982).

It is also interesting to note the correlation between the pH optimum of the chlamydial MurA activity and the pH of the chlamydial inclusion. Schramm *et al* found that at 2 hpi, the pH of inclusions formed by *C. trachomatis* serovar L2 is neutral and drops to a pH of 6.26 at 4 hpi before leveling at pH 6.6 at 12 hpi (Schramm *et al.*, 1996). Because of the method used to measure the pH, they were unable to measure the pH after 12 hours. Recently, Grieshaber *et al* were able to measure the pH of the chlamydial

inclusion at 20 hpi and reported a pH of 7.25 (Grieshaber *et al.*, 2002). We suggest that the C→D change in the active site of the chlamydial MurA is the result of evolutionary pressure to select a variant enzyme with optimal activity within the unique environment of the chlamydial inclusion. Our data suggest that the chlamydial MurA is required during early stages of the chlamydial life cycle, when EBs convert to RBs and RBs begin to expand in size and divide. At these times, the pH of the inclusion is such that MurA is optimally active. Later in the life cycle, the environment of the inclusion potentially changes, which may render the chlamydial MurA less active, to accommodate other events. Perhaps *Chlamydia* are able to recycle PG, therefore needing less MurA during late stages of the life cycle.

FOS is a broad-spectrum antibiotic that irreversibly binds to the active site of MurA (Kahan *et al.*, 1974; Wu and Venkateswaran, 1974). FOS forms a covalent adduct with a cysteine residue in the active site of MurA, thus rendering the enzyme inactive (Kahan *et al.*, 1974; Marquardt *et al.*, 1994). In *E. coli*, replacement of the cysteine in the active site of MurA with aspartate renders the bacteria resistant to FOS (Kim *et al.*, 1996). *Mycobacterium tuberculosis* is innately resistant to FOS and *M. tuberculosis* MurA is resistant to FOS *in vitro*. Sequence analysis of the *M. tuberculosis murA* revealed a cysteine to aspartate change in the MurA active site. Engineering an aspartate to cysteine change in the active site of *M. tuberculosis* MurA renders the enzyme sensitive to FOS *in vitro* (De Smet *et al.*, 1999). Our sequence analysis of *C. trachomatis* serovar L2 MurA predicted a similar C→D change in the active site. *C. trachomatis* serovar D and *C. pneumoniae* MurA sequences also contain an aspartate residue instead of cysteine in the active site of the enzyme (Read *et al.*, 2000; Stephens *et al.*, 1998). In this work, we have

demonstrated that, similar to *M. tuberculosis*, *Chlamydia* spp. are innately resistant to FOS. Although we have shown that FOS can enter the eukaryotic cell and effectively kill the intracellular pathogen, *Shigella*, we cannot rule out that *Chlamydia* is innately resistant to FOS due to the inability of the drug to enter the inclusion. However, it has been shown that the pore size of the chlamydial inclusion lies between 45 and 520 Da (Grieshaber *et al.*, 2002; Heinzen and Hackstadt, 1997). FOS, which is approximately 185 Da, is small enough to pass through this pore. Therefore, we do not believe that the innate resistance to FOS is due to an inability of the drug to reach *Chlamydia.* Rather, FOS resistance in *Chlamydia* can be attributed to the activity of the chlamydial MurA. Correlating with our *in vivo* resistance data, the *in vitro* chlamydial MurA activity was uninhibited at high concentrations of FOS. Resistance to high concentrations of FOS was also conferred to an *E. coli ΔmurA* mutant expressing the chlamydial *murA.* Although overexpression of the FOS-sensitive *E. coli* MurA in ecMurA increased the resistance to FOS (Figure 1.6B), this strain was still sensitive to high concentrations of FOS. However, ctMurA was resistant to high concentrations of FOS, suggesting that the activity of the chlamydial MurA accounted for the resistance of ctMurA. FOS is generally not used to treat chlamydial infections. Therefore, we believe it is reasonable to conclude that the C→D conversion in the active site of the chlamydial MurA is not the result of antibiotic pressure to select for a FOS resistant variant of *Chlamydia*. Rather, evolutionary selective pressure for an enzyme better adapted to the environment of the chlamydial inclusion is the likely explanation for this alteration in the active site residue.

Within 2 hours after infection by *C. trachomatis*, EBs begin to convert to RBs. A size and volume increase in the RBs becomes evident 8-12 hpi. This increase continues

over several hours. Around 12 hpi, RBs begin dividing by binary fission. After 18 hpi, increasing numbers of RBs begin differentiating back to EBs, while others continue to divide. Approximately 36-72 hpi, the cells lyse and EBs are released to initiate another round of infection (Hackstadt, 1999). Our RT-PCR data suggest that PG is involved in size expansion of RBs and chlamydial cell division. *Chlamydia murA* mRNA transcripts are expressed shortly after the eukaryotic cells are infected with EBs while there is a six hour lag before *murB* mRNA transcripts are expressed. Shaw *et al* reported that *murG* (Figure 1.1) was not expressed at 6 hpi but was expressed at 12 hpi (Shaw *et al.*, 2002). We propose that the expression of mRNA transcripts from each PG pathway gene differs in relation to its place in the pathway and the developmental cycle. *Chlamydia murA* mRNA is expressed as early as EBs begin converting to RBs possibly generating a pool of UDP-GlcNAc enolpyruvate. Approximately 6 hours later, *murB* mRNA and potentially other PG genes are expressed allowing UDP-MurNAc and all or part of the attached pentapeptide to be generated. Between 6 and 12 hpi, *murG* and possibly other PG genes are expressed and the disaccharide-pentapeptide subunit is synthesized and flipped to the periplasm. In the periplasm, penicillin-binding proteins crosslink the subunits, giving rise to PG layers that expand the size and volume of RBs and facilitate cell division. Further support for this model are the expression of *pbp2* mRNA transcripts 6-8 hpi (data not shown) and the effectiveness of cell wall antibiotics against *Chlamydia*, which interfere with RB development and cell division.

Chlamydial genomes are relatively small and for the most part encode pathways to synthesize compounds that the organism cannot acquire from the host. Therefore, from an evolutionary point of view, it is highly unlikely that chlamydiae would retain a nearly

complete pathway for PG synthesis if these genes did not encode functional products. Because nearly all the PG synthesis homologues are present in *Chlamydia*, it is unlikely that the PG synthesis enzymes are involved in other cellular processes. *Chlamydia* lack both the alanine and glutamine racemase, Alr and MurI, respectively, for a complete PG synthesis pathway. It has been speculated that *Chlamydia* could acquire these D-amino acids from the host. However, there does not appear to be conclusive evidence to support this idea. More likely, *Chlamydia* encodes a racemase(s) that is not similar to racemases in other bacteria. The presence of D-alanine in chlamydial PG is supported by the sensitivity of *Chlamydia* to D-cycloserine, which inhibits the D-alanine ligase.

Many recent publications have indirectly suggested that the question of PG in *Chlamydia* needs to be reevaluated. For example, synthesis of MurC/DdlA was identified in a proteome analysis of *C. pneumoniae* EBs, while MurG was detected in a combined genomic-proteomic analysis of surface exposed proteins in *C. pneumoniae* EBs (Montigiani *et al.*, 2002; Vandahl *et al.*, 2001). It should be noted that previous attempts to detect PG have been performed with purified EBs. Our data strongly suggest that not only is PG capable of being synthesized in *Chlamydia* but that the presence of PG in *Chlamydia* should be reanalyzed in RBs. Biochemical evidence of PG in *Chlamydia* will be of significance in that it would open up a new avenue to the treatment of chlamydial infections through the application of pre-existing and novel drugs that target the chlamydial PG synthesis

Chapter 2.1

CHARACTERIZATION OF *CHLAMYDIA* MURC-DDL, A FUSION PROTEIN EXHIBITING D-ALANYL-D-ALANINE LIGASE ACTIVITY INVOLVED IN PEPTIDOGLYCAN SYNTHESIS AND D-CYCLOSERINE SENSITIVITY: *ESCHERICHIA COLI* AS A HETEROLOGOUS HOST

SUMMARY

Recent characterization of chlamydial genes encoding functional peptidoglycan (PG)-synthesis proteins suggests that the *Chlamydiaceae* possess the ability to synthesize PG yet biochemical evidence for the synthesis of PG has yet to be demonstrated. The presence of D-amino acids in PG is a hallmark of bacteria. *Chlamydiaceae* do not appear to encode amino acid racemases however a D-alanyl-D-alanine (D-Ala-D-Ala) ligase homologue (Ddl) is encoded in the genome. Thus, we undertook a genetics-based approach to demonstrate and characterize the D-Ala-D-Ala ligase activity of chlamydial Ddl, a protein encoded as a fusion with MurC. The full-length *murC-ddl* fusion gene from *Chlamydia trachomatis* serovar L2 was cloned and placed under the control of the arabinose-inducible *ara* promoter and transformed into a D-Ala-D-Ala ligase auxotroph of *Escherichia coli* possessing deletions of both the *ddlA* and *ddlB* genes. Viability of the *E. coli* Δ*ddlA* Δ*ddlB* mutant in the absence of exogenous D-Ala-D-Ala dipeptide became dependent on the expression of the chlamydial *murC-ddl* thus demonstrating functional ligase activity. Domain mapping of the full-length fusion protein and site directed

mutagenesis of the MurC domain revealed that the structure of the full fusion protein but not MurC enzymatic activity was required for ligase activity *in vivo*. Recombinant MurC-Ddl exhibited substrate specificity for D-Ala. *Chlamydia* growth is inhibited by D-cycloserine (DCS) and *in vitro* analysis provided evidence for the chlamydial MurC-Ddl as the target for DCS sensitivity. *In vivo* sensitivity to DCS could be reversed by addition of exogenous D-Ala and D-Ala-D-Ala. Together, these findings further support our hypothesis that PG is synthesized by members of the *Chlamydiaceae* family and suggest that D-amino acids, specifically D-Ala, are present in chlamydial PG.

INTRODUCTION

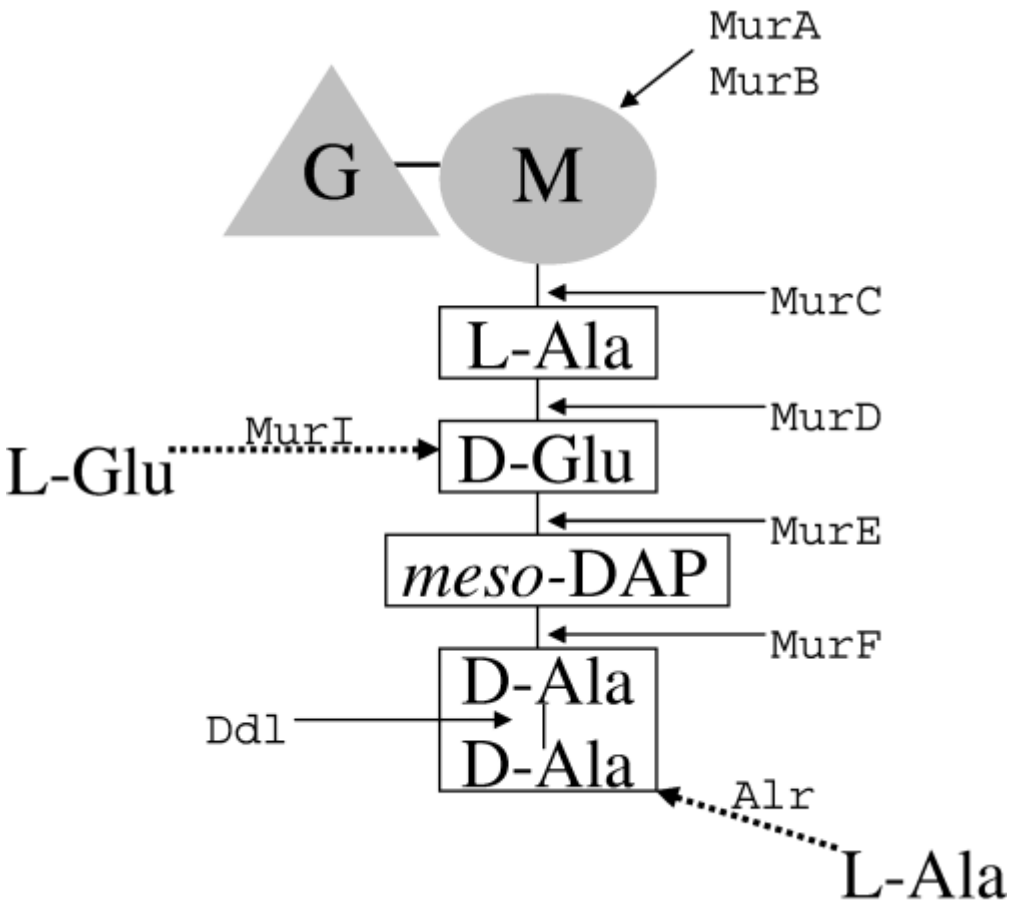
Chlamydiaceae are obligate, intracellular, gram-negative-like pathogens that cause significant ocular, sexually transmitted, and respiratory infections worldwide. Recent taxonomic evidence has divided members of the *Chlamydiaceae* family into two distinct genera, *Chlamydia* and *Chlamydophila* (Bush and Everett, 2001). For simplicity, we refer to both genera as *Chlamydia.* *Chlamydia* have an atypical biphasic developmental cycle (Wyrick, 2000). Infectious yet metabolically inert elementary bodies (EBs) survive in the extracellular environment to attach to and enter host epithelial cells. After entry into the host cell, the bacteria reside in a specialized phagosome in the host cell cytoplasm termed the inclusion. In the inclusion, EBs begin to differentiate into metabolically active reticulate bodies (RBs) which divide by binary fission and later differentiate back to EBs. Late in the developmental cycle, the host cell is lysed, releasing EBs, RBs and intermediate bodies. Released EBs can initiate subsequent rounds of infection in surrounding cells.

Morphologically, *Chlamydia* resemble typical gram-negative bacteria. Electron microscopy of both EBs and RBs clearly identifies a cytoplasmic membrane and outer membrane. The cell envelope lipid and amino acid compositions of *Chlamydia* are also similar to other gram-negative organisms and the presence of lipopolysaccharide in the outer membrane has been detected. However, unlike all other gram-negative bacteria, *Chlamydia* lack detectable levels of peptidoglycan (PG) (Chopra *et al.*, 1998; Fox *et al.*, 1990). The absence of detectable PG in *Chlamydia* remains a paradox as the bacteria are sensitive to cell wall inhibitors such as β-lactams and D-cycloserine (DCS), synthesize penicillin-binding proteins (PBPs) and encode a nearly complete pathway for the synthesis of PG (Chopra *et al.*, 1998; Moulder, 1993).

In nearly all gram-negative bacteria, the basic building block of PG is identical, containing the D-amino acids D-alanine (D-Ala) and D-glutamate (D-Glu) and the unique saccharide, muramic acid. The basic structure of PG is also assembled by homologous enzymes (van Heijenoort, 2001) (Figure 2.1.1). Recently, we demonstrated for the first time the functionality of an enzyme in the PG synthesis pathway of *Chlamydia* and suggested that this pathway is functional in making PG subunits in *Chlamydia* (McCoy *et al.*, 2003). Our notion is further supported by Hesse *et al*. who reported UDP-*N*-acetylmuramate:amino acid ligase activity encoded by chlamydial *murC* and by chlamydial proteomic studies that detected MurC-Ddl and MurG in EBs (Hesse *et al.*, 2003; Montigiani *et al.*, 2002; Vandahl *et al.*, 2001). While it now appears that *Chlamydia* has the capacity to synthesize PG, the question remains as to the chemical composition of the chlamydial PG, specifically the presence of D-amino acids.

In nature, amino acids are found almost exclusively as the L-enantiomer thus bacteria possess racemases that convert the L- to the D-enantiomer. Genome sequencing failed to reveal genes in *Chlamydia* that encode alanine or glutamate racemases, however *Chlamydia* do encode a D-Ala-D-Ala ligase homologue (Ddl) (Read *et al.*, 2000; Read *et al.*, 2003; Shirai *et al.*, 2000; Stephens *et al.*, 1998; Thomson *et al.*, 2005). Moreover all *Chlamydia* encode Ddl as a fusion with MurC (MurC-Ddl). This gene fusion is exclusively found in all members of the *Chlamydiaceae* family. In bacteria that synthesize PG, Ddl is essential and the ligation of two D-Ala residues by Ddl proteins is highly specific. The addition of D-Ala-D-Ala to PG monomers is crucial to the survival of bacteria as the pentapeptide chains of the PG monomers are cross-linked in the periplasm at the penultimate D-Ala of one peptide chain to diaminopimelic acid in another chain.

Figure 2.1.1. Cytoplasmic PG-monomer synthesis pathway in gram-negative bacteria. The basic PG monomer in gram-negative bacteria is synthesized in the cytoplasm as a disaccharide-pentapeptide, which contains a unique sugar (muramic acid) and D-amino acids. Components of the pentapeptide are boxed and enzymes for PG synthesis and assembly are linked by arrows to the step at which they act. Dotted arrows represent enzymes that are missing from *Chlamydia*. G=*N*-acetylglucosamine, M=*N*-acetylmuramic acid


MurA
MurB
G
M
MurC
L-Ala
MurD
MurI
L-Glu
D-Glu
MurE
meso-DAP
MurF
D-Ala
Ddl
D-Ala
Alr
L-Ala

Mechanistically important amino acid residues have been conserved in Ddl homologues identified in many organisms (Fan *et al.*, 1994). This conservation of essential amino acids extends to *Chlamydia* Ddl and suggests functionality and specificity of *Chlamydia* Ddl. *Chlamydia* are also sensitive to DCS, a substrate analogue of D-Ala that inhibits alanine racemase and D-Ala-D-Ala ligase (Lin and Moulder, 1966; Moulder *et al.*, 1963). In the absence of an alanine racemase, the sensitivity of *Chlamydia* to DCS predicts that Ddl is a functional D-Ala-D-Ala ligase and would be the target of DCS.

In this report, we show that MurC-Ddl from *C. trachomatis* functions as a D-Ala-D-Ala ligase by complementing a conditional lethal double deletion of *ddlA* and *ddlB* in the heterologous host *Escherichia coli*. Furthermore, *in vivo* D-Ala-D-Ala ligase activity depends upon the structure of the full fusion protein but not the enzymatic activity of MurC. *In vitro* assay of recombinant MurC-Ddl ligase activity confirmed D-Ala as the substrate for ligation. *In vivo*, DCS inhibits growth and spread of *Chlamydia*, which can be reversed in the presence of D-Ala and D-Ala-D-Ala but not other D- or L-amino acids. *In vitro* ligase activity confirmed MurC-Ddl as a target of DCS. These data further support our hypothesis that *Chlamydia* can synthesize a PG-like structure and predict that PG in *Chlamydia* contains D-amino acids. In the absence of an alanine racemase homologue(s), *Chlamydia* spp. must therefore acquire or generate D-amino acids by a novel pathway.

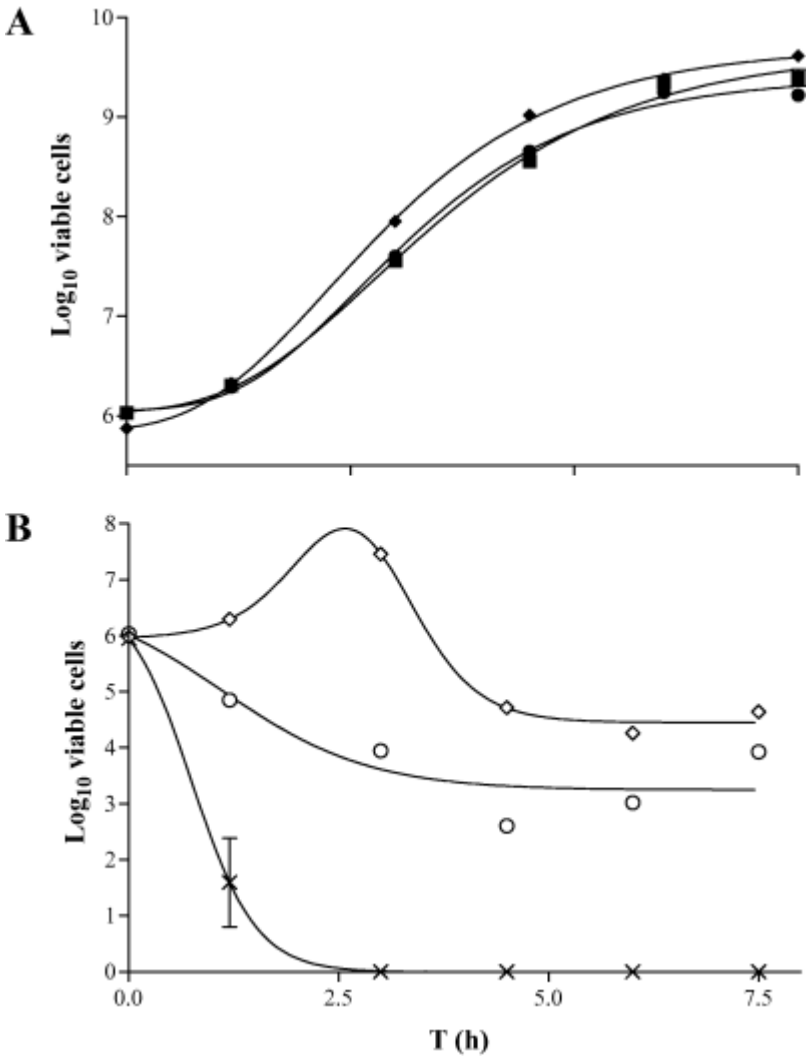
RESULTS

C. trachomatis murC-ddl *encodes a functional D-Ala-D-Ala ligase.*

To assess the activity of chlamydial MurC-Ddl, we first generated a D-Ala-D-Ala ligase mutant of *E. coli* (Experimental Procedures). *E. coli* encodes two *ddl* genes (*ddlA* and *ddlB*). A mutation in either gene that renders the Ddl protein inactive is not lethal to *E. coli*, however mutations in both genes render the bacteria auxotrophic for D-Ala-D-Ala as the dipeptide is essential for PG synthesis (Figure 2.1.2B). ATM718 (*E. coli* Δ*ddlA* Δ*ddlB::kan*) was transformed with pAJM35 or pAJM38 expressing the D-Ala-D-Ala ligase gene of *C. trachomatis* (*murC-ddl*$_{Ct}$) and *E. coli* (*ddlA*$_{Ec}$), respectively, under the control of the arabinose-inducible, glucose-repressible *ara* promoter. Transformants were selected on M9 minimal glucose medium containing ampicillin and D-Ala-D-Ala dipeptide and subsequently screened for the ability to grow on LB arabinose agar medium in the absence of exogenous D-Ala-D-Ala. Expression of either *ddlA*$_{Ec}$ or *murC-ddl*$_{Ct}$ in the presence of arabinose was sufficient to support growth of ATM718 on agar in the absence of D-Ala-D-Ala. The colony phenotype of both mutants appeared identical in size and morphology (data not shown).

To evaluate the growth kinetics and demonstrate arabinose-dependent growth of ATM718 expressing *murC-ddl*$_{Ct}$ (CT762), the number of viable bacteria was determined under inducing and repressing conditions. When the cloned genes were expressed, the number of viable ATM718 expressing *murC-ddl*$_{Ct}$ was the same as the number of viable ATM718 expressing *ddlA*$_{Ec}$ and wild-type *E. coli* (*ddlA*$^{+}$ *ddlB*$^{+}$) (Figure 2.1.2A). As expected, viability was dependent upon the arabinose-inducible expression of the D-Ala-D-Ala ligases (Figure 2.1.2B). A complete loss in viability under glucose-repressing

Figure 2.1.2. *murC-ddl*$_{Ct}$ complementation in *E. coli* Δ*ddlA* Δ*ddlB::kan*. A. ATM718 (*E. coli* Δ*ddlA ⊿ddlB::kan*) *containing* pAJM35 (*murC-ddl*$_{Ct}$;circles) and pAJM38 (*ddlA*$_{Ec}$;diamonds) were grown overnight in LB Amp arabinose and subcultured into medium containing arabinose. At specified times, dilutions of the cultures were plated onto LB arabinose to determine the number of viable bacteria. Platings were performed in triplicate and compared to wild-type *E. coli* BL21(DE3) (squares). B. ATM718 containing pAJM35 (circles) and pAJM38 (diamonds) were grown and plated as in (A) except that the overnight culture was subcultured into medium containing glucose. Platings were performed in triplicate and compared to BL21(DE3) Δ*ddlA* Δ*ddlB::kan* (×).


A
10
9
8
7
6
Log10 viable cells
B
8
7
6
5
4
3
2
1
0
Log10 viable cells
0.0
2.5
5.0
7.5
T (h)

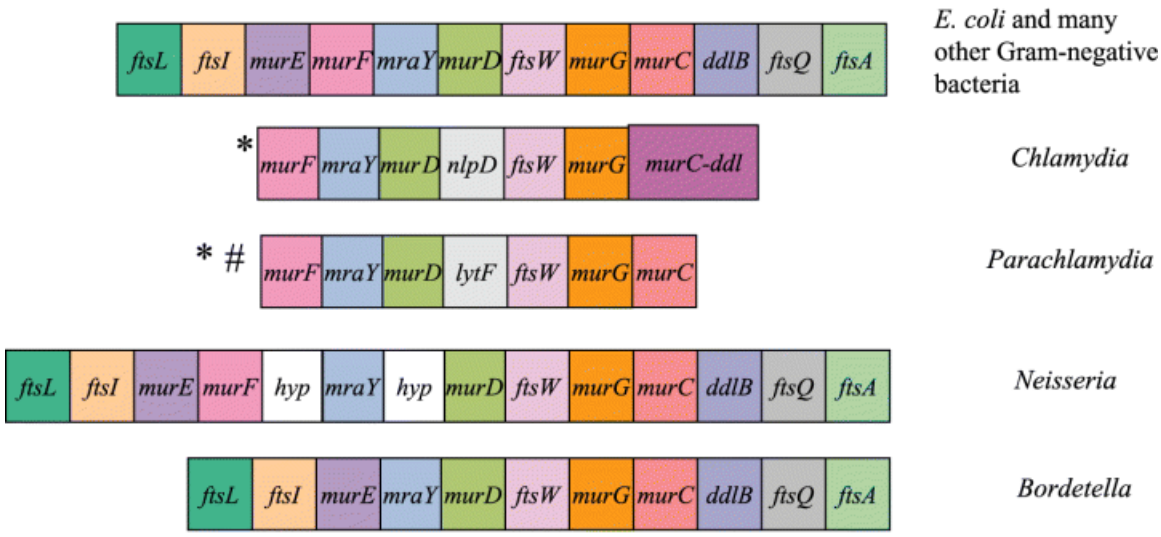
conditions was not observed with the complemented mutants compared to the complete loss of viability of ATM718 in the absence of D-Ala-D-Ala. This result was probably due to the leaky expression of the *ara* promoter under repressing conditions in this system.

D-Ala-D-Ala ligase activity depends upon the structure of MurC-Ddl.

In many gram-negative organisms, *murC* is found directly upstream of a *ddl* gene in an operon termed the *dcw* gene cluster (Miyakawa *et al.*, 1972; van Heijenoort, 2001). Genes in the *dcw* gene cluster encode proteins involved in both PG synthesis and cell division (Figure 2.1.3). All *Chlamydia* genomes sequenced thus far have retained much of this gene organization however *murC* and *ddl* are found as a fused gene in *Chlamydia* but in no other bacterial species (Read *et al.*, 2000; Read *et al.*, 2003; Shirai *et al.*, 2000; Stephens *et al.*, 1998; Thomson *et al.*, 2005). We cloned and sequenced *murC-ddl*$_{Ct}$ from *C. trachomatis* serovar L2. The encoded MurC-Ddl from *C. trachomatis* serovar L2 shared 99% similarity to *C. trachomatis* serovar D, 89% to *C. muridarum*, 76% to *C. cavaie*, and 72% to *C. pneumoniae* MurC-Ddl using PBLAST alignment (www.ncbi.nlm.nih.gov/BLAST). The MurC domain of MurC-Ddl from *C. trachomatis* serovar L2 shared 61% similarity to MurC from *Parachlamydia* and 51% to MurC from *E. coli* while the Ddl domain shared 41%, 50% and 53% similarity to Ddl from *Parachlamydia* and DdlA and DdlB from *E. coli*, respectively.

This *Chlamydia*-specific fusion led us to ask whether MurC might be required for Ddl activity. To address this question we generated a hypothetical *E. coli* MurC-DdlB fusion *in silico* and aligned it with MurC-Ddl from *C. trachomatis* in order to identify the separation point of the chlamydial MurC and Ddl domains, which appeared to be at amino

Figure 2.1.3. ***dcw*** **(division/cell wall synthesis) gene cluster in representative gram-negative organisms.**

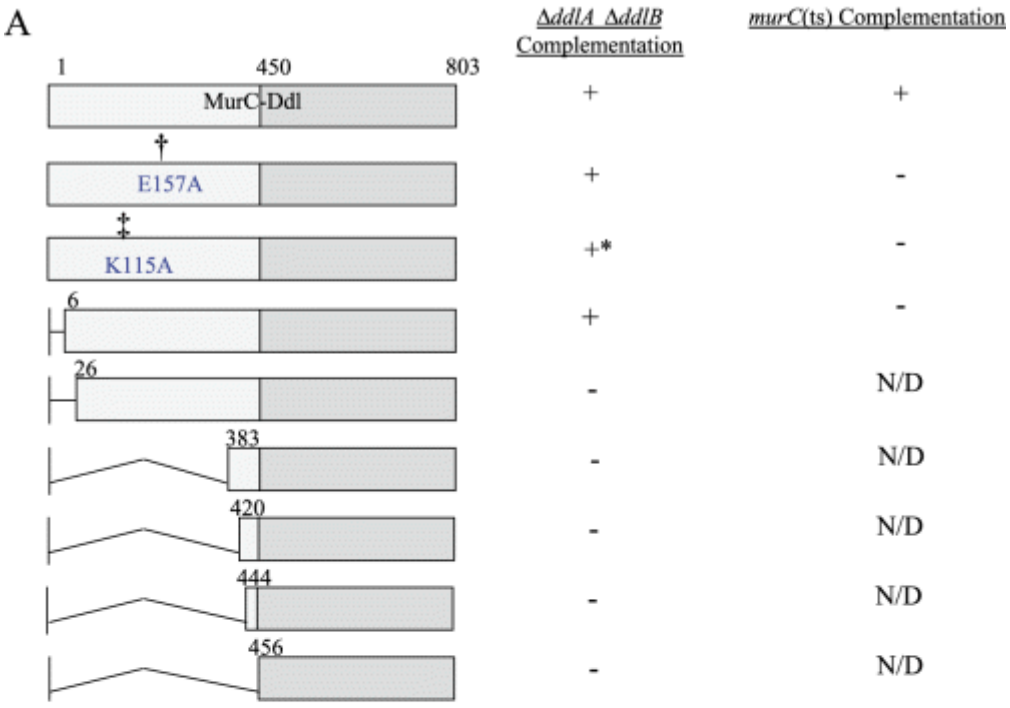


* *ftsI* and *murE* are together in another region of the genome
\# *ddl* is in another region of the genome

acid 450. Subcloning and expression of different MurC-deletion constructs of MurC-Ddl in ATM718 was unable to restore viability to ATM718 in the absence of D-Ala-D-Ala on either LB agar or M9 agar (Figure 2.1.4A; data not shown). The smallest portion of the MurC domain that could be deleted and still allow MurC-Ddl to retain D-Ala-D-Ala ligase activity was the first five N-terminal amino acids. Deletion of these amino acids rendered the MurC domain enzymatically inactive. Control experiments showed that the MurC deletion constructs containing a C-terminal 6-His tag were expressed at comparable levels as wild-type MurC-Ddl containing a C-terminal 6-His tag (data no shown). Thus, the N-terminal MurC domain is required for the functionality of the C-terminal Ddl domain.

Because expression of MurC-Ddl_{6-803} was able to complement Ddl activity but not MurC activity *in vivo*, we wanted to confirm that the enzymatic activity of the MurC domain was not required for function of the Ddl domain. We employed site-directed mutagenesis to inactivate MurC while leaving the structure of the fusion protein intact. MurC, MurD, MurE and MurF belong to the Mur synthetase family of non-ribosomal synthesis proteins. Sequence alignment of the Mur synthetase proteins from many organisms has identified 11 invariant amino acids and a mutation at one of these amino acids (E174A) in MurC of *E. coli* eliminates the activity of the enzyme both *in vivo* and *in vitro* (Bouhss *et al.*, 1997). Interestingly, a change in the invariant lysine (K130A) of the Walker A box greatly reduces the activity of *E. coli* MurC, but still complements a *murC3*(ts) defect albeit with morphological defects (Bouhss *et al.*, 1997). Inclusion of the chlamydial MurC in the alignment of Mur synthetases reveals that all 11 invariant amino acids are present in the protein sequence of MurC-Ddl (Figure 2.1.4B, data not shown). Based on these results, we chose to make alanine replacements of the invariant K115 and

Figure 2.1.4. Domain mapping and amino acid changes in MurC-Ddl. (A) *C. trachomatis murC-ddlA* containing 5' deletions of, or site directed mutations in the *murC* domain were cloned into pBAD18 and expressed in *E. coli* Δ*ddlA* Δ*ddlB::kan.* After selection, the transformants were screened for growth on LB agar in the absence of D-Ala-D-Ala. K115A represents an alanine substitution for an invariant lysine in the MurC Walker box A; E157A represents an invariant glutamic acid to alanine change. Mutation of the respective invariant amino acids in *E. coli* MurC alters the activity of the enzyme (Bouhss *et al.*, 1997). * = growth was supported in the absence of exogenous D-Ala-D-Ala however, in culture the bacteria grew in a biofilm. Amino acid 450 represents the separation point of the two chlamydial domains based on alignment of chlamydial MurC-Ddl with an *in silico* fusion of MurC and DdlB from *E. coli*. (B) Partial alignment of the Mur synthetases from *E. coli* and *C. trachomatis* corresponding to *Chlamydia* MurC E157 and K115 amino acid regions . Invariant amino acids are highlighted in gray and the Walker A box is underlinded. Note that the amino acid in the sixth position of the Walker A box (shaded red) can be either S or T. N/D = not determined



B

```
MurC-ecoli  121  GIAIAGTHGKTTTTAMVS
MurC-ctrac  106  SIFVTGSHGKTTVSSLIT
MurD-ecoli  106  IVAITGSNGKSTVTTLVG
MurD-ctrac  103  SLAITGTTGKTTILFLE
MurE-ecoli  110  LVGVTGTNGKTTTQLLA
MurE-ctrac  104  VIGITGTNGKTTVSCLVR
MurF-ecoli  102  VVALTGSSGKTSVKEMTA
MurF-ctrac  106  VIGITGSVGKTTTKNFAN

MurC-ecoli  170  TLIAEADESDASF-------LHLQPMVA--IVTNIEADHMDTY
MurC-ctrac  153  YFVAEADESDGSI-------RCYTPEFS--VITNIDDEHLSNF
MurD-ecoli  153  LYVLELSSFQLET------TSSLQAVAA--TILNVTEDHMDRY
MurD-ctrac  149  VRIVEISSFQLAD------QEKSYPVLSGGMILNISDNHLD-Y
MurE-ecoli  178  FCAMEVSSHGLVQ----HRVAALKFAAS--VFTNLSRDHLDYH
MurE-ctrac  172  AAVMEVSSIGMAL----GRVRETEFLAG--VLTNITSDHLDFH
MurF-ecoli  154  YAVIELGANHQGE--IVWTVSLTRPEAA--LVNNLAAAHLEGF
MurF-ctrac  158  FLLLEMGVSEPNN--MKNLLEIIEPTIG--VITHIDVQHAVHF
```

E157 residues in the MurC domain of chlamydial MurC-Ddl and assess the ligase activity *in vivo*. K115 and E157 of chlamydial MurC correspond to K130 and E174 of *E. coli* MurC, respectively (Figure 2.1.4B). First, we assessed the activity of $MurC_{K115A}$-Ddl and $MurC_{E157A}$-Ddl by complementation in ST222 (*E. coli* $murC_{ts}$). At the non-permissive temperature, expression of wild-type MurC-Ddl fully complemented while expression of $MurC_{K115A}$-Ddl and $MurC_{E157A}$-Ddl in ST222 were unable to rescue the viability defect (Figure 2.1.4A).

After verifying that the site-directed MurC-Ddl mutations abolished *in vivo* MurC activity, we asked whether the D-Ala-D-Ala ligase activity of $MurC_{K115A}$-Ddl and $MurC_{E157A}$-Ddl was still functional. Expression of the chlamydial $MurC_{E157A}$-Ddl in ATM718 supported growth on agar and in broth in the absence of D-Ala-D-Ala and no obvious growth or morphological defects were observed on plates and in broth. Similarly, expression of chlamydial $MurC_{K115A}$-Ddl in ATM718 was sufficient to support growth on LB arabinose agar in the absence of D-Ala-D-Ala, however the colonies were very sticky and when inoculated into LB arabinose broth, the bacteria grew as a large mass rather than as individual cells. Together, these data suggest that the Ddl activity is dependent upon the structure of the fusion with MurC but not upon MurC synthetase activity.

In vitro assay of D-Ala-D-Ala ligase activity.

The MurC-Ddl fusion in *Chlamydia* is unique and specific to this family of bacteria. Interestingly, the preferential substrate for both MurC and Ddl proteins is alanine albeit in different enantiomer form. Ddl enzymes specifically utilize D-Ala as a substrate, while MurC enzymes, including chlamydial MurC, add L-Ala to muramic acid (Hesse *et*

al., 2003; Park, 1996; van Heijenoort, 2001). Thus, we asked whether the presence of Ddl as a fusion with MurC in *Chlamydia* altered the substrate specificity of the Ddl ligase activity. An alteration in Ddl substrate specificity might explain the absence of a dedicated alanine racemase in the chlamydial genomes. However, as revealed in Figure 2.1.5, recombinant MurC-Ddl specifically catalyzed the synthesis of D-Ala-D-Ala. Recombinant MurC-Ddl ligase activity was optimal over a range of pH 6 to 8 as indirectly measured by the release of inorganic phosphate (data not shown).

Next, recombinant MurC-Ddl was characterized kinetically using an ADP release coupled reaction described previously (Daub *et al.*, 1988). D-Ala-D-Ala ligases bind two D-Ala molecules in two distinct binding sites thus two K_m values for D-Ala can be obtained (Zawadzke *et al.*, 1991). When we analyzed MurC-Ddl under steady state conditions, we found that K_1 (N-terminal D-Ala binding site) was in the low μM range compared to K_2 (C-terminal D-Ala binding site) which was approximately 450-fold higher (Table 2.1.1). These values were comparable to those published for *Salmonella* DdlA and *E. coli* DdlA and DdlB (Zawadzke *et al.*, 1991). Likewise, the K_m for ATP was also within range of that reported for *Salmonella* and *E. coli* D-Ala-D-Ala ligases. However, the k_{cat} for MurC-Ddl was very high (2.8 to 6.5-fold higher than that for *Salmonella* and *E. coli* Ddl proteins). This apparently large turn-over value may be skewed by the large molecular weight of the enzyme being a fusion protein.

Minimal inhibitory concentration (MIC) of DCS against C. trachomatis serovar L2.

One of the paradoxes of *Chlamydia* biology is the sensitivity of *Chlamydia* to cell wall targeting antibiotics such as DCS in the absence of a detectable cell wall. Recently, our laboratory has developed a method for determining minimal inhibitory concentrations

Figure 2.1.5. ***In vitro*** **MurC-Ddl ligase activity and amino acid substrate specificity.** Recombinant MurC-Ddl protein was incubated for 3 hours at 37°C in the presence of different D- and L-amino acids. Reactions were spotted on a cellulose TLC plate, eluted, dried and stained with ninhydrin. (1) D-Ala-D-Ala, (2) D-Ala, (3) L-Ala, (4) D-Ser, (5) enzyme alone, (6), enzyme + D-Ala, (7), enzyme + L-Ala, and (8) enzyme + D-Ser.

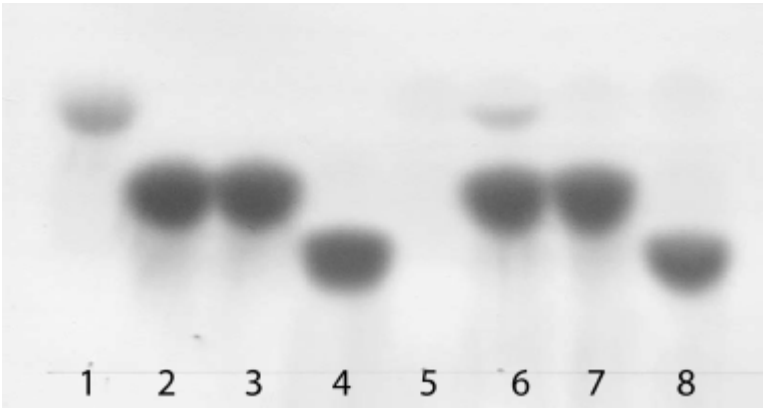

1 2 3 4 5 6 7 8

Table 2.1.1. Kinetic Properties of MurC-Ddl as a D-Ala-D-Ala Ligase.

Substrate/ inhibitor		*E. coli* DdlA[a]	*E. coli* DdlB[a]	*Chlamydia* MurC-Ddl
D-Alanine	*k*cat (min^{-1})	444	1018	2908[b]
	K_1 (μM)	5.7	3.3	1.2
	K_2 (mM)	0.55	1.2	0.55
ATP	K_m (μM)	38	40	17
DCS	K_i (μM)	8.9 (C)	27 (C)	2 (C)

[a] Data for *E. coli* DdlA and DdlB are from Zawadske *et. al.,* 1991. C = competitive. [b] k_{cat} was calculated based on the molecular weight of the full MurC-Ddl fusion protein.

(MIC) for antibiotics that inhibit *Chlamydia* growth by use of a chlamydial plaque assay and 10^5 plaque forming units (pfu) of *Chlamydia* (Banks *et al.*, 1970; Binet and Maurelli, submitted). Rather than observation of inclusions per microscopic field, the basis of the plaque assay is the formation of plaques in a confluent monolayer of eukaryotic cells. Using the plaque assay, the antimicrobial activity of a drug can be assessed against a standard *Chlamydia* input that corresponds to that used to determine MICs against bacteria that replicate extracellularly (*i. e.* 10^5 colony forming units). This is an important aspect of determining the MIC of a drug against *Chlamydia* as studies have noted the effect of inoculum size on the susceptibility of an organism, including *Chlamydia*, to a drug (Gordon and Quan, 1973). Using this method, we determined the MIC of DCS against our strain of *C. trachomatis* serovar L2 and determined that plaque formation of 10^5 pfu was completely inhibited in the presence of 30 μg/ml DCS (data not shown).

Moulder *et al* first reported that DCS inhibited the growth of *C. psittaci* in chick embryo sacs (Moulder *et al.*, 1963). This study also demonstrated that growth inhibition by DCS could be reversed in the presence of exogenous D-Ala or a racemic mixture of DL-Ala-DL-Ala but not L-Ala or other D-amino acids. To verify that the same reversal of growth inhibition by DCS occurs in *C. trachomatis* serovar L2, we evaluated the ability of 10^5 *C. trachomatis* EBs to form plaques in the presence of 30 μg/ml DCS and various molar equivalents of exogenous amino acids. As seen in Table 2.1.2, the addition of a one-tenth molar equivalent of exogenous D-Ala or an equivalent molar concentration of D-Ala-D-Ala to DCS reversed some but not all of the plaque formation inhibition by DCS, while 1X and 10X molar equivalents of exogenous D-Ala or 10X molar equivalent of D-Ala-D-Ala completely reversed the DCS inhibition. While the addition of one-tenth and 1X molar

Table 2.1.2. Effect of amino acids on DCS inhibition of *Chlamydia* growth *in vivo*.

DCS, μg/ml	Amino acid, AA:DCS ratio	Plaque formation
0	--	++++
30	--	-
	D-Ala, 1:10	+++
	D-Ala, 1:1	++++
	D-Ala, 10:1	++++
	L-Ala, 1:10	-/+
	L-Ala, 1:1	-/+
	L-Ala, 10:1	+
	D-Ser, 1:10	-/+
	D-Ser, 1:1	-/+
	D-Ser, 10:1	+
	D-Ala-D-Ala, 1:10	++
	D-Ala-D-Ala, 1:1	+++
	D-Ala-D-Ala, 10:1	++++

C. trachomatis serovar L2 was grown in the presence of DCS (30 μg/ml) and varying molar equivalent concentrations of D-Ala, L-Ala, D-Ser and D-Ala-D-Ala as described in *Experimental Procedures*. (++++), complete infection and lysis of the monolayer; (+++), numerous large plaques but less than complete lysis of the monolayer; (++), numerous very small plaques; (+), few (~100) small plaques; (-/+), less than 10 pinpoint plaques/dish ; (-), no plaque formation.

equivalent of D-Ala-D-Ala could reverse some of the DCS inhibition, D-Ala-D-Ala was not as effective as D-Ala at the same molar equivalent. Neither exogenous D-Ser nor L-Ala at 10-fold higher amino acid:DCS ratios could reverse the inhibitory effects of DCS against *Chlamydia*.

Chlamydia MurC-Ddl is a target of DCS.

DCS is a substrate analogue of D-alanine and an effective inhibitor of both alanine racemase and D-Ala-D-Ala ligase (Neuhaus and Hammes, 1981). DCS irreversibly inactivates alanine racemases while reversibly inhibiting D-Ala-D-Ala ligases. Genome sequencing of different *Chlamydia* spp. failed to reveal a dedicated alanine racemase homologue, therefore the logical chlamydial target of DCS is MurC-Ddl. To verify this assumption, we determined the K_i for DCS. DCS competitively inhibits the D-Ala-D-Ala ligase activity of MurC-Ddl *in vitro*, with a K_i value of 2 μM (Table 2.1.1). This low K_i value is similar to those reported for *Salmonella* and *E. coli* Ddl proteins (Zawadzke *et al.*, 1991) and suggests that DCS preferentially binds to the N-terminal D-Ala binding site.

DISCUSSION

The current lack of a genetic manipulation system in *Chlamydia* as well as the obligate intracellular niche these organism occupy have hampered our understanding of many aspects of *Chlamydia* biology. However, these hindrances have been offset by the recently available genome sequences of seven strains representing four species of *Chlamydia* (Read *et al.*, 2000; Read *et al.*, 2003; Shirai *et al.*, 2000; Stephens *et al.*, 1998; Thomson *et al.*, 2005). Despite the inability to perform genetic manipulations in *Chlamydia*, knowledge of the unique biology of these organisms has been gained by using established genetic systems of heterologous host systems such as *E. coli* to identify the function of both homologous and *Chlamydia*-specific gene products (Hesse *et al.*, 2003; Karunakaran *et al.*, 2003; McCoy *et al.*, 2003; Schwoppe *et al.*, 2002; Sweet *et al.*, 2001; Tipples and McClarty, 1995; Wylie *et al.*, 1997; Zhang *et al.*, 1995). Phylogenetically, *Chlamydia* lie very distant to other eubacteria thus the ability of a chlamydial gene to complement a homologous mutation in another eubacteria provides a strong indication that the gene product expressed in *Chlamydia* is functional.

One aspect of *Chlamydia* biology that has been greatly enhanced by complementation cloning in a heterologous host system has been our understanding of chlamydial PG synthesis. The existence of PG in *Chlamydia* is a paradox. It has been known for many years that *Chlamydia* are sensitive to antibiotics that target PG and they synthesize penicillin binding proteins (PBP), however PG has yet to be detected in *Chlamydia.* Thus it was a surprise when genome sequencing revealed a nearly complete pathway for the synthesis of PG both as monomeric subunits in the cytoplasm (Figure 2.1.1) and as a polymer in the periplasm (Chopra *et al.*, 1998; Ghuysen and Goffin, 1999).

Recent biochemical and genetic studies using *E. coli* have demonstrated the *in vitro* and *in vivo* functionality of both MurA and MurC (Figure 2.1.1) from *C. trachomatis*, suggesting that the chlamydial PG synthesis pathway is functional (Hesse *et al.*, 2003; McCoy *et al.*, 2003). As visualized in Figure 2.1.2, the number of chlamydial genes that encode for functional PG-synthesis proteins is now expanded to include *ddl*.

The functionality of Ddl in *Chlamydia* is interesting and unique in many ways. All PG whether from gram-negative or gram-positive organisms contain D-Ala. The penultimate D-Ala of PG pentapeptide chains plays a key role in the cross-linking of PG subunits by forming a bond with the third amino acid in an adjacent PG pentapeptide chain. This cross-linking provides rigidity to the PG polymer (van Heijenoort, 2001). Because of the vital role of D-Ala in establishing the integrity of PG, bacteria encode dedicated racemases that convert L-Ala to D-Ala. In the case of *Chlamydia,* all chlamydial genomes sequenced thus far are devoid of amino acid racemases, yet chlamydial Ddl specifically ligates two D-Ala residues to form D-Ala-D-Ala (Figure 2.1.5). The question arises as to how *Chlamydia* obtain D-Ala. It has been speculated that *Chlamydia* obtain D-amino acids from the eukaryotic host or another exogenous source via DagA, a transporter of glycine, D-Ala, D-serine. While high levels of D-serine and D-aspartate have been found in the brain and D-amino acid containing peptides have been occasionally isolated from invertabrates, there is no strong evidence that eukaryotic cells contain D-amino acids (Chopra *et al.*, 1998; Dunlop *et al.*, 1986; Ghuysen and Goffin, 1999; Hasimoto *et al.*, 1992; Kreil, 1997). Moreover, racemase mutants of invasive *Listeria* and *Shigella* cannot survive in host eukaryotic cells unless exogenous D-Ala is added to the medium (Thompson *et al.*, 1998); McCoy and Maurelli, unpublished data). We suggest that

Chlamydia utilizes a novel pathway to synthesize D-Ala and we are currently attempting to identify this pathway.

The structure of *E. coli* DdlB has been determined and, based upon the crystal structure, eleven amino acids have been proposed to be involved in the catalytic mechanism of *E. coli* DdlB (Fan *et al.*, 1994). Of these eleven, ten have been confirmed by site-directed mutagenesis to play a role in ligase activity (Shi and Walsh, 1995). Interestingly, D-Ala-D-Ala ligases are related to a group of proteins (Van proteins) that play a role in vancomycin resistance in gram-positive bacteria. These proteins catalyze the synthesis of D-Ala-D-Ser and D-Ala-D-Lactate. Extensive alignment of the deduced amino acid sequences of Ddl and Van proteins from many different organisms revealed that the ten amino acids involved in the activity of *E. coli* DdlB are strictly conserved in all Ddl and Van proteins (Evers *et al.*, 1996). When we included the amino acid sequences of the Ddl domain of MurC-Ddl from all four *Chlamydia* spp. sequenced thus far, we found that these ten amino acids are also conserved throughout the *Chlamydiaceae.* The fact that all *Chlamydia* spp. have maintained these amino acids suggests that the gene sequences are not artifactual remnants from a chlamydial ancestor and the encoded gene products likely function in chlamydiae to synthesize PG. Our experimental data reported here confirm this prediction for MurC-Ddl.

The fact that the MurC and Ddl proteins are encoded as a fusion in the *Chlamydiaceae* family is intriguing. Rarely in an operon do mutations occur such that two genes fuse and encode one bifunctional product. In the case of chlamydiae this gene fusion has maintained the activities of both the MurC and Ddl domains (Hesse *et al.*, 2003). It is unclear as to why this fusion of *murC* and *ddl* occurred during evolution. The recently

published genome sequence of the obligate intracellular amoebal symbiont *Parachlamydia* UWE25 revealed that this ancestor of *Chlamydia* not only encodes separate proteins for both MurC and Ddl but *murC* and *ddl* are unlinked (Figure 2.1.3). The *Parachlamydia* genome also encodes an alanine racemase (Horn *et al.*, 2004). This suggests that the fusion of *murC* and *ddl* and the loss of the racemase in *Chlamydiaceae* as well as the translocation of *ddl* in *Parachlamydia* occurred during the evolution to pathogenic *Chlamydia.* It is possible that the fusion of MurC and Ddl in the *Chlamydiaceae* family has allowed the protein to acquire a third function. What that function might be remains to be determined although one attractive possibility is that MurC-Ddl has gained alanine racemase activity. We believe this possibility is unlikely as neither expression of chlamydial *murC-ddl* in an *E. coli* Δ*dadX* Δ*alr::kan* D-Ala auxotroph on LB agar nor expression of chlamydial *murC-ddl* in an invasive *Shigella* Δ*dadX* Δ*alr::kan* D-Ala auxotroph in the plaque assay restore viability in the absence of exogenous D-Ala (McCoy and Maurelli, unpublished data).

Hesse *et al* separated the MurC domain from the Ddl domain and clearly demonstrated that the MurC domain functions as a UDP-*N*-acetylmuramyl:amino acid ligase both *in vivo* and *in vitro* (Hesse *et al.*, 2003). Contrary to the MurC domain, when we physically separated the two domains, Ddl lost its ability to function as a D-Ala-D-Ala ligase (Figure 2.1.4A). It is likely that the pathway by which *Chlamydia* obtains D-Ala is not as efficient as a dedicated racemase and thus the concentration of D-Ala in the chlamydial cytoplasm may be minimal. Accordingly, one can envisage that the fusion of MurC and Ddl as well as the dependence of the Ddl enzymatic activity on the structure of the full fusion protein have occurred in order to restrict the synthesized D-Ala-D-Ala dipeptide in close proximity to the PG monomer as it is being synthesized.

Chlamydia spp. are among the most prevalent bacterial pathogens, causing significant disease in both humans and animals. *C. trachomatis* is the most commonly reported bacterial sexually transmitted infection in the United States as well as the primary cause of preventable infectious blindness worldwide. *C. pneumoniae* infections, which appear to occur in healthy individuals several times during their lifetime without any signs of infection, have been associated with chronic diseases such as Alzheimer's disease and atherosclerosis (Belland *et al.*, 2004; Grayston, 2000). Future studies to determine the structure of this unique MurC-Ddl fusion protein would be of value for the development of *Chlamydia*-specific antibiotics that target and disrupt the junction between the two domains.

While we speculated that the substrate specificity for the Ddl domain of MurC-Ddl may have been altered due to its fusion with the L-Ala adding enzyme, MurC, we were not surprised to observe the high specificity for D-Ala (Figure 2.1.5). Antimicrobial susceptibility studies using antibiotics that target cell-wall synthesis in *Chlamydia* strongly suggested that D-Ala plays a role in chlamydial biology. Penicillin and β-lactam antibiotics inhibit transpeptidation, a process that involves the pentultimate D-Ala of the PG pentapeptide chain and PBPs. Chlamydiae are susceptible to penicillin and β-lactam antibiotics and three PBPs have been identified in chlamydial EBs and RBs based on their ability to bind radiolabeled penicillin (Barbour *et al.*, 1982). An alteration in substrate specificity of the Ddl domain such that L-Ala-L-Ala is synthesized would require an alteration in substrate specificity of the chlamydial PBPs as well as MurF. From an evolutionary standpoint, these alterations would not be feasible.

Although not used clinically, DCS is a substrate analogue of D-Ala that inhibits the function of Ddl proteins. For over fourty years, it has been known that the psittacosis group of *Chlamydia* is sensitive to DCS (Moulder *et al.*, 1963). Like the psittacosis group, *C. trachomatis* is also sensitive to DCS (Table 2.1.2). Although the assays used here and by Moulder (the plaque assay and embryonic yolk sacs, respectively) to determine the MIC of DCS that inhibits chlamydial growth are distinctly different, the MICs are relatively the same, and in both cases, chlamydial growth inhibition by DCS is reversed by the addition of exogenous D-Ala or D-Ala-D-Ala dipeptide but not other amino acids (Table 2.1.2). While Moulder *et al.* could only speculate that DCS inhibited growth by blocking cell wall synthesis in *Chlamydia,* our work demonstrates that DCS does indeed block PG synthesis in *Chlamydia* by competitively inhibiting the enzymatic activity of Ddl (Table 2.1.1). Because DCS also inhibits the activity of alanine racemases, we cannot rule out the possibility that DCS also targets the mechanism by which *Chlamydia* obtains D-Ala.

Because DCS and D-Ala are competitive substrates for Ddl while D-Ala-D-Ala is the product catalyzed by Ddl, it was expected that D-Ala-D-Ala would reverse DCS growth inhibition *in vivo* at lower molar equivalents than D-Ala. However, using the plaque assay, we observed the opposite effects (Table 2.1.1). This difference may be at the level of competition for transport into the cell. Chlamydiae encode two homologues of DagA/CycA, which transports D-Ala, D-Ser, and glycine. In *E. coli* DCS is also transported into the cell by DagA/CycA (Neuhaus and Hammes, 1981). In addition, D-Ala-D-Ala transport into cells occurs by di- and oligopeptide permeases. Thus, it is likely that in the presence of exogenous D-Ala competition occurs at the level of the transporter and less DCS is transported into *Chlamydia* ultimately raising the apparent MIC of DCS.

In contrast the transport of DCS in *Chlamydia* is probably not affected by exogenous D-Ala-D-Ala. Because a greater molar concentration of D-Ala-D-Ala is required to reverse DCS growth inhibition, the kinetics of D-Ala-D-Ala uptake in *Chlamydia* is likely slower than the kinetics of D-Ala uptake. In any case, these data suggest that D-Ala and formation of D-Ala-D-Ala plays an essential role in *Chlamydia* biology.

While the goal of our work was to characterize chlamydial Ddl, we needed to assess chlamydial MurC activity during some of our experiments. Sequence alignment of Mur synthetases (MurC, MurD, MurE and MurF) identified 11 invariant amino acids common to all of these enzymes (Bouhss *et al.*, 1997). By aligning the MurC, MurD, MurE and MurF protein sequences with the homologous *E. coli* proteins, we observed that all 11 invariant amino acids are conserved in chlamydial MurC and MurE while 10 of the 11 invariant amino acids are conserved in chlamydial MurD and MurF sequences (Figure 2.1.3, data not shown). In all sequenced bacteria of genus *Chlamydia* the invariant amino acid that is different as well as the amino acid that is found at this position is the same. In the case of *E. coli* MurC, two of the 11 invariants are known to play a crucial role in the activity of the enzyme (Bouhss *et al.*, 1997). As seen in Figure 2.1.3, the respective invariant amino acids are essential for chlamydial MurC activity. The fact that all members of the *Chlamydiaceae* family have maintained these invariant amino acids further supports the notion that the gene sequences have been maintained in *Chlamydia* to encode functional PG synthesis enzymes. Together with the demonstration that the chlamydial MurC domain functions as a UDP-*N*-acetylmuramyl:amino acid ligase in *E. coli* (Hesse *et al.*, 2003), our data further support the notion that PG synthesis occurs in *Chlamydia*. Our data also suggest that the PG monomer assembled in *Chlamydia* is of the same chemical structure as

PG monomers in other gram-negative bacteria and contains D-amino acids (Figure 2.1.1). Further understanding as to the mechanism by which *Chlamydia* obtains D-amino acids, the structure of chlamydial MurC-Ddl and the biochemical detection of chlamydial PG would significantly contribute to treatment of chlamydial infections through the use of pre-existing and novel antimicrobials that target PG synthesis in *Chlamydia.*

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions.

C. trachomatis serovar L2 was kindly provided by Harlan Caldwell (Rocky Mountain Laboratories, Hamilton, MT). *E. coli* strains used in this study are listed in Table 2.1.3. Strains were grown at 37ºC in Luria-Bertani (LB) or M9 minimal medium with aeration or on agar. When appropriate, medium was supplemented with ampicillin (100 μg/ml), kanamycin (50 μg/ml), chloramphenicol (15 μg/ml), arabinose (0.1%), glucose (0.2%) or D-Ala-D-Ala (400 μg/ml). D-Ala-D-Ala and DCS were purchased from Sigma Chemical Co. (St. Louis, MO).

ATM718 (*E. coli* Δ*ddlA* Δ*ddlB*::*kan*) was created by sequential application of the one-step gene inactivation method as described (Datsenko and Wanner, 2000). BW25113 containing pKD46 was the transformation recipient for all mutant construction. A P1L4 lysate was grown on the Δ*ddlA* and Δ*ddlB* strains and used to transduce *E. coli* BL21(DE3). First, the Δ*ddlA::kan* allele was transduced into *E. coli* BL21(DE3). The Kan^R transductant was then transformed with pCP20, which expresses FLP and mediates recombination between FRT sequences, ultimately excising the *kan* cassette. Next, the Δ*ddlB::kan* allele was transduced into the Kan^S *E. coli* Δ*ddlA* strain and Kan^R transductants were selected on M9 minimal agar medium containing kanamycin and D-Ala-D-Ala. Resulting transductants were screened for D-Ala-D-Ala auxotrophy and a double mutant was stocked as ATM718.

Construction of murC-ddl expression vectors.

pAJM35 and pAJM38 contain an optimized *E. coli* RBS and complete open reading frame of the *murC-ddl* and *ddlA* genes from *C. trachomatis* serovar L2 and *E. coli* K-12,

Table 2.1.3. Strains and Plasmids.

Strains and Plasmids	Relevant Genotype	Source or Reference
Strains		
BL21(DE3)	*E. coli* B F^- *ompT hsdS*(r_B^- m_B^-) *dcm* Tet^+ *gal* λ (DE3) *endA* Hte	Stratagene
BL21(DE3) pLysS	*E. coli* B F^- *ompT hsdS*(r_B^- m_B^-) *dcm met gal* (DE3) pLysS (Cam^R)	Novagen
ATM697	BL21(DE3) pLysS transformed with pAJM54	This work
ATM718	BL21(DE3) Δ*ddlA* Δ*ddlB::kan*	This work
ATM719	BL21(DE3) pAJM38/ Δ*ddlA* Δ*ddlB::kan*	ATM718 transformed with pAJM38
ATM720	BL21(DE3) pAJM35/Δ*ddlA* Δ*ddlB::kan*	ATM718 transformed with pAJM35
ATM721	BL21(DE3) pAJM35(K115A)/ Δ*ddlA* Δ*ddlB::kan*	ATM718 transformed with pAJM35(K115A)
ATM722	BL21(DE3) pAJM35(E157A)/ Δ*ddlA* Δ*ddlB::kan*	ATM718 transformed with pAJM35(E157A)
ST222	*E. coli thr-1 araC14 leuB6*(AM) *murC3*(ts) *secA216 fhuA61 lacY1 galT23* λ^- *trp-84 his-215 thyA710 rpsL263 xylA5 mtl-1 thi-1*	(Miyakawa *et al.*, 1972)
Plasmids		
pBAD18	Arabinose-inducible expression vector, Amp^R, $pMB1_{ori}$, moderate copy number	(Guzman *et al.*, 1995)
pET19b	N-terminal His-Tag fusion expression vector, Ap^R	Novagen
pAJM35	pBAD18::*murC-ddl*$_{Ct}$; coding sequence from *C. trachomatis* serovar L2	This work
pAJM35(K115A)	K115A site-directed mutant of pAJM35	This work
pAJM35(E157A)	E157A site-directed mutant of pAJM35	This work
pAJM37	pBAD18::*murC-ddl*$_{Ct}$(383-803) ; deletion of the N-terminal amino acids 1-382 from ORF pAJM35	This work
pAJM38	pBAD18::*ddlA*$_{Ec}$; coding sequence from BL21(DE3)	This work
pAJM44	pBAD18::*murC-ddl*$_{Ct}$(420-803) ; deletion of the N-terminal amino	This work

	acids 1-419 from ORF pAJM35	
pAJM54	pET19b::*murC-ddl*$_{Ct}$	This work
pAJM59	pBAD18::*murC-ddl*$_{Ct}$(26-803) ; deletion of the N-terminal amino acids 1-25 from ORF pAJM35	This work
pAJM62	pBAD18::*murC-ddl*$_{Ct}$(444-803) ; deletion of the N-terminal amino acids 1-443 from ORF pAJM35	This work
pAJM63	pBAD18::*murC-ddl*$_{Ct}$(456-803) ; deletion of the N-terminal amino acids 1-455 from ORF pAJM35	This work

respectively, under the control of the *ara* promoter of pBAD18. pBAD18 was chosen for its arabinose-inducible glucose-repressible regulation of gene expression.

The *murC-ddl*$_{Ct}$ gene (CT762) was PCR amplified from *C. trachomatis* serovar L2 genomic DNA using Platinum *Taq* polymerase (Invitrogen, Carlsbad, CA). The upstream primer (OAM61; 5'-AGGAGGAATTCACC*ATG*ATGAAAAGCTTGTTTTACCACT-3') contained an *E. coli* optimized RBS (underlined) prior to the start codon (italicized), whereas the downstream primer (OAM62; 5'-ATGCGTCGAC**TTA**CTGAATAGCGAACGCTTGATCTACAA-3') contained a SalI restriction site (underlined) and the stop codon (bold). Primer design was based on the genome sequence of *C. trachomatis* serovar D (http://chlamydia-www.berkeley.edu:4231/). The PCR fragment was gel purified, digested with SalI and ligated into SmaI/SalI digested pBAD18. The same strategy was used to clone the *ddlA* gene from *E. coli* BL21(DE3). The upstream and downstream *E. coli ddlA* primers (OAM65; 5'-AGGAGGAATTCACC*ATG*GAAAAACTGCGGGTAGGAATCGTTTTTG-3' and OAM66; 5'-ATGCGTCGAC**TTA**CATTGTGGTTTTCAATGCGTTATCCGCAGCGT-3', respectively) contain the same RBS and SalI restriction site as described for *murC-ddl*$_{Ct}$.

Primers OAM68 (5'-AGGAGGAATTCACC*ATG*GAGGCGGAAGATATCTCGTACCA-3'), OAM80 (5'-AGGAGGAATTCACC*ATG*CGTGTACACGATGTGTGTGTATCTTTA-3'), OAM135 (5'-AGGAGGAATTCACC*ATG*TTTTACCACTTTATTGGTATTGGTGGGATTGG-3'), OAM139 (5'-AGGAGGAATTCACC*ATG*GATCGAGGATATAGCGTATCGGGAAG-3'), OAM149 (5'-AGGAGGAATTCACC*ATG*GAGCCTCAAAAACTGCACTTAG-3'), and OAM150 (5'-AGGAGGAATTCACC*ATG*GGGAAATCATGCGAACAC-3') which

contains an *E. coli* optimized RBS (underlined) and an artificial start codon (italicized), was used with OAM62 to make a 5' deletion of *murC-ddl*$_{Ct}$, specifically bp 1-1146, 1-1257, 1-15, 1-75, 1-1329, and 1-1365, respectively. The mutant *murC-ddl*$_{Ct}$ gene was cloned into pBAD18 as described above and gave rise to pAJM37, pAJM44, pAJM55, pAJM59, pAJM62 and pAJM63, respectively.

Site-directed mutagenesis based on the QuikChange Site-Directed Mutagenesis procedure (Stratagene, La Jolla, CA) was employed to change the MurC domain invariant amino acids K115 and E157 to alanine. Briefly, primers OAM108 (5'-AGAGACTGTTGTCGCCCCATGACTTCCTGT-3') and OAM109 (5'-ACAGGAAGTCATGGGGCGACAACAGTCTCT-3') or OAM131 (5'-TTGTTGCTGCAGCAGATGAAAGCG) and OAM132 (5'-CGCTTTCATCTGCTGCAGCAACAA-3') were used to amplify pAJM35 and introduce the K115A and E157A mutations into the expressed MurC-Ddl protein. Base changes are underlined. After DpnI-treatment, PCR amplified products were transformed into DH5α. Ampicillin-resistant transformants were screened by sequencing for the correct base change. One correct clone was selected and digested with SacI and HpaI. The resulting 1.1 kb fragment was ligated into the 5.9 kb SacI, HpaI-digested pAJM35. Again, ampicillin-resistant clones were screened by sequencing to insure the integrity of the DNA sequence and the resulting plasmids were designated pAJM35(K115A) and pAJM35(E157A). Sequencing of all cloned genes was performed by the Biomedical Instrumentation Center at USUHS.

Viability curves

Cultures were grown overnight in the presence of arabinose before being diluted into 12.5 ml of LB containing appropriate antibiotics and arabinose (or glucose) in order to give an initial OD_{600} reading of 0.001. Cultures were grown at 37°C with aeration and dilutions of the samples were plated on LB containing arabinose such that 30 to 300 colony forming units (cfu) were obtained per plate.

Domain mapping of MurC-Ddl

pAJM37, pAJM35(K115A), pAJM35(E157A), pAJM44, pAJM59, pAJM62 and pAJM63 were transformed into ATM718 and transformants were selected on M9 glucose minimal medium containing ampicillin, kanamycin and D-Ala-D-Ala. Transformants were subsequently streaked on LB agar containing ampicillin, kanamycin and arabinose. Complementation was assessed by the arabinose-dependent growth of transformed ATM718 in the absence of D-Ala-D-Ala.

Protein expression and purification

The wild-type *murC-ddl*$_{Ct}$ gene was PCR amplified using OAM115 (5'-GATCGGATCCTTACTGAATAGCGAACGCTTGA-3')/OAM116 (5'-GATC*CATATG*ATGATGAAAGCTTGTTTTACCACT-3'), which introduce NdeI (italicized) and BamHI (underlined) restriction sites. After digestion, the PCR products were introduced into pET19b (Novagen, Madison, WI) in order to express the protein with an N-terminal His-tag. Expression and purification of the recombinant protein was carried

out according to the protocol for the Ni-NTA purification system (Invitrogen, Carlsbad, CA).

Thin Layer Chromatography (TLC) Assay of D-Ala-D-Ala ligase activity

Assays (10 μl) contained 100 mM HEPES, pH 8.0, 10 mM KCl, 10 mM $MgCl_2$, 5 mM ATP, 10 mM amino acid and recombinant MurC-Ddl (10 μM) and were incubated at 37ºC for 3 hours. Two μL of each reaction were spotted onto a cellulose TLC plate, eluted for 4 hours in 1-butanol-acetic acid-water (12:3:5), dried, and stained with 0.2% ninhydrin.

Kinetic Analysis of D-Ala-D-Ala ligase activity

Steady-state enzyme kinetic assays were performed using a continuous ADP release coupled assay as previously described (Daub *et al.*, 1988). The decrease in NADH absorbance at 340 nm was monitored.

Assay of plaque formation by Chlamydia

MIC and reversal of DCS-sensitivity assays were performed using varying concentrations of DCS and amino acids in a plaque assay (Binet and Maurelli, submitted). Confluent monolayers of L2 mouse fibroblast cells in 60 mm^2 dishes were incubated for 120 minutes at 37ºC and 5% CO_2 with 10^5 plaque forming units of *C. trachomatis* serovar L2 (MOI~0.01). After infection, infection medium was removed and the eukaryotic cells were overlayed with a low-melting point agarose medium (0.25%) containing Dulbecco's Modified Eagle Medium (1X), fetal bovine serum (10%), non-essential amino acids (1X), cycloheximide (200 ng/ml), gentamicin (20 μg/ml) and varying concentrations of DCS and

amino acids. At 7 days post-infection a second agarose overlay was added to each dish. At 12-14 days post infection, plaque formation was visualized by staining the cells for 4 hours at 37ºC and 5% CO_2 with 0.5% neutral red.

Chapter 2.2

CHARACTERIZATION OF *CHLAMYDIA* MURC-DDL, A FUSION PROTEIN EXHIBITING D-ALANYL-D-ALANINE LIGASE ACTIVITY INVOLVED IN PEPTIDOGLYCAN SYNTHESIS AND D-CYCLOSERINE SENSITIVITY: *SHIGELLA FLEXNERI* AS A HETEROLOGOUS HOST

SUMMARY

The existence of peptidoglycan (PG) in *Chlamydia* is a paradox as these bacteria are sensitive to antibiotics that target cell-wall synthesis and encode a nearly complete pathway for the synthesis of PG despite the fact that PG has yet to be detected biochemically. To date, three chlamydial PG synthesis genes have been characterized using the heterologous host system of *Escherichia coli*. In this work, we set out to establish whether the facultative intracellular *Shigella flexneri* would be a better heterologous host system for the characterization of chlamydial PG synthesis genes by characterizing the D-alanyl-D-alanine (D-Ala-D-Ala) ligase activity of the *C. trachomatis* MurC-Ddl. Expression of the *C. trachomatis* serovar L2 *murC-ddl* gene in a Δ*ddlA* Δ*ddlB*::*kan* D-Ala-D-Ala auxotroph of *S. flexneri* 2a was sufficient to restore viability to the mutant in the absence of exogenous D-Ala-D-Ala in LB. However, expression of the chlamydial *murC-ddl* in the *Shigella* D-Ala-D-Ala auxotrophic mutant reduced the ability of *Shigella* to invade mammalian cells. Furthermore, of the bacteria that were able to invade when the chlamydial *murC-ddl* was expressed, no cell-to-cell spread via actin polymerization was observed and the intracellular viability of the *Shigella* was rapidly lost. These mutants also

display morphological defects both in LB and intracellularly, which likely contribute to the invasion and intracellular growth phenotypes that we observe. Comparing these results to those reported using the heterologous host system of *E. coli*, we believe that (1) expression of *murC-ddl* in *Shigella* may provide new insight into differences in the PG synthesis pathway of *E. coli* and *Shigella* and (2) these differences support the notion that *Shigella* may not be the best heterologous host system for the characterization of chlamydial PG synthesis genes.

INTRODUCTION

Currently, *Chlamydia* lacks a system of genetic manipulation. Consequently, genetically characterizing the activity of chlamydial gene products requires the use of a heterologous host system. A handful of chlamydial gene products have been characterized in *E. coli* by complementation cloning (Hesse *et al.*, 2003; McCoy *et al.*, 2003; McCoy and Maurelli, 2005; Schwoppe *et al.*, 2002; Sweet *et al.*, 2001; Tipples and McClarty, 1995; Zhang *et al.*, 1995). However, most *E. coli* are extracellular bacteria while *Chlamydia* occupies a strictly intracellular niche. Because of this lifestyle, the activity of chlamydial enzymes may be altered due to host cell factors or the overall unique environment of the mammalian cell. Thus, greater knowledge of chlamydial gene products may be obtained using a heterologous host system in an intracellular environment. To address this notion, we proposed that *Shigella* may be better suited as a heterologous host for the characterization of chlamydial gene products.

Shigellae are facultative intracellular pathogens. After invading epithelial cells, *Shigella* undergoes replication in the host cytosol and polymerizes host actin to facilitate their spread to neighboring epithelial cells. Using *Shigella* rather than another facultative intracellular organism offers a few advantages. First, *Shigella* is genetically very closely related to *E. coli.* Indeed, genetic alterations constructed in *E. coli* can be readily moved into *Shigella* by transduction. Also, numerous assays exist to evaluate the different steps of invasion, intracellular growth and cell-to-cell spread of *Shigella.* Moreover, the ability of *Shigella* to grow extracellularly as well as intracellularly offers the ability to directly compare the effect of expression of chlamydial genes in the two different environments. Because *Shigella* and *E. coli* are so closely related genetically, we can also compare the

effect that expression of chlamydial genes has on the pathogen versus a commensal organism.

With this in mind, we set out to characterize the D-Ala-D-Ala ligase activity of the *C. trachomatis* MurC-Ddl when it was expressed in intracellularly versus extracellularly grown *Shigella.* Like *E. coli,* two D-Ala-D-Ala ligase genes, *ddlA* and *ddlB,* are present in the *Shigella* genomes. In *E. coli,* the loss of either *ddlA* or *ddlB* is not lethal to the bacteria. However the loss of both genes renders the organism auxotrophic for D-Ala-D-Ala dipeptide. Viability can be restored to the *E. coli* double D-Ala-D-Ala ligase mutant if either $ddlA_{Ec}$ or $murC\text{-}ddl_{Ct}$ is expressed *in trans* (McCoy and Maurelli, 2005). Using mutations that were created in *E. coli,* we constructed BS775, a Δ*ddlA* Δ*ddlB::kan* mutant of *S. flexneri* 2a. Viability of BS775 grown in LB was dependent upon the expression of either $murC\text{-}ddl_{Ct}$ or $ddlA_{Ec}$ *in trans.* However, invasion of HeLa cells by BS775 expressing the $murC\text{-}ddl_{Ct}$ was drastically reduced. Furthermore, the intracellular bacteria displayed morphology defects and rapidly lost viability. The morphological defects exhibited by the intracellular BS775 expressing the $murC\text{-}ddl_{Ct}$ were more pronounced when the strain was grown overnight in LB broth. Furthermore, these defects do not appear to be linked to the expression of the type III secretion (T3S) system. Based upon what is known regarding the chlamydial MurC-Ddl activity in *E. coli,* these results suggest that *Shigella* either is not a good heterologous host system to express chlamydial PG synthesis genes or can reveal previously unidentified properties of the D-Ala-D-Ala ligase activity of the chlamydial MurC-Ddl.

RESULTS

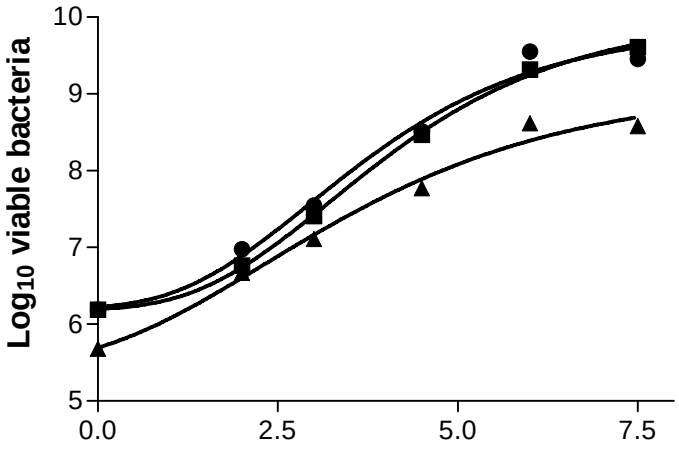
murC-ddl *from* C. trachomatis *encodes a functional D-Ala-D-Ala ligase in Shigella grown extracellularly.*

To ascertain the functionality of the D-Ala-D-Ala ligase activity of the *murC-ddl*$_{Ct}$ gene product during conditions of intracellular bacterial growth, we constructed a Δ*ddlA* Δ*ddlB::kan* mutant of *S. flexneri* 2a (2457T). The strategy used is described in the Experimental Methods section in Chapter 2.1. Briefly, P1L4 lysates were grown on ATM714 (*E. coli* Δ*ddlA::kan*) and ATM715 (*E. coli* Δ*ddlB::kan*). First, the Δ*ddlA::kan* mutation was transduced into 2457T. The resulting Kan^R mutant (BS773) was then transformed with pCP20, which expresses a yeast Flp recombinase. The Flp recombinase facilitates a recombination event between two FRT sequences resulting in the loss of the DNA between the FRT sequences. In BS773, expression of the FLP recombinase leads to a recombination event between FRT sequences flanking the *kan* gene ultimately giving rise to a 2457T Δ*ddlA* mutant that is kanamycin sensitive (BS774). BS774 was finally transduced to kanamycin resistance with the P1L4 lysate grown on ATM715. One transductant was selected and stored as BS775.

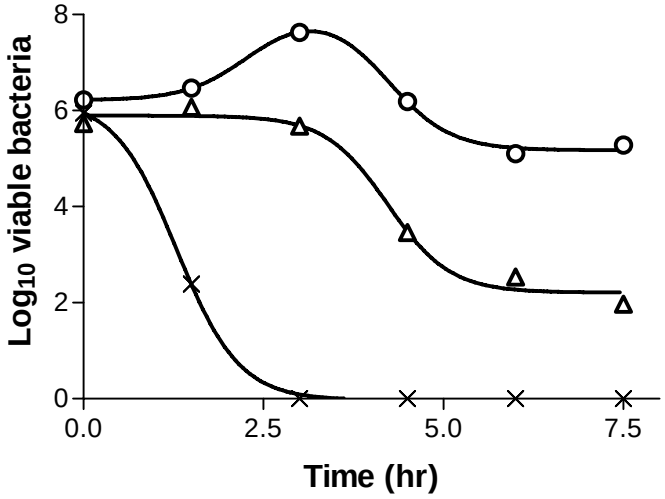
We first determined the viability of BS775 expressing *murC-ddl*$_{Ct}$ and *ddlA*$_{Ec}$ from the regulated *ara* promoter in pBAD18 when grown in LB. In the absence of exogenous D-Ala-D-Ala, the viability of BS775 was only restored when the expression of either *murC-ddl*$_{Ct}$ or *ddlA*$_{Ec}$ was induced (*i. e.* in the presence of arabinose) (Figure 2.2.1). Interestingly, as the culture of BS775 expressing *murC-ddl*$_{Ct}$ reached stationary phase, there was an approximately one-log lower number of viable bacteria/mL compared to 2457T (Figure 2.2.1A). We did not compare the viability of BS775 expressing *ddlA*$_{Ec}$ or

Figure 2.2.1. Ability of *murC-ddl*$_{Ct}$ to restore viability to BS775. Complementation of the D-Ala-D-Ala auxotrophy of BS775 by *murC-ddl*$_{Ct}$ in pBAD18 was determined under arabinose-inducing (filled symbols) and glucose-repressing (open symbols) conditions. A. Wild-type 2457T (square) and BS775 transformed with *ddlA*$_{Ec}$ (circle) or *murC-ddl*$_{Ct}$ (triangle) were grown O/N in LB with arabinose and subcultured in LB with arabinose. At various times post subculture, samples were diluted and plated onto LB agar containing arabinose. The number of cfu/mL was calculated and the $\log_{10}$ cfu/mL was plotted versus time (hr). B. BS775 (X) and BS775 transformed with *ddlA*$_{Ec}$ (circle) or *murC-ddl*$_{Ct}$ (triangle) were grown O/N in M9 minimal medium with glucose and D-Ala-D-Ala (BS775) or LB with arabinose (BS775 containing pBAD vectors) and subcultured in LB medium containing glucose. At various times post subculture, samples were diluted and plated onto M9 minimal medium with glucose and supplemented with D-Ala-D-Ala (BS775) or LB agar medium containing arabinose (BS775 containing pBAD vectors). The number of cfu/mL was calculated and the $\log_{10}$ cfu/mL was plotted versus time (hr).

A


Log10 viable bacteria
10
9
8
7
6
5
0.0
2.5
5.0
7.5

B


Log10 viable bacteria
8
6
4
2
0
0.0
2.5
5.0
7.5
Time (hr)

murC-ddl$_{Ct}$ to that of BS775 alone grown in LB supplemented with D-Ala-D-Ala as BS775 only grows in M9 minimal medium supplemented with D-Ala-D-Ala. While it is clear that the viability of BS775 depends upon expression of *murC-ddl*$_{Ct}$ and *ddlA*$_{Ec}$ genes *in trans*, we were unable to completely inhibit growth under glucose-repressing conditions (Figure 2.2.1B). This lack of full repression when using the *ara* promoter has been noted previously (McCoy and Maurelli, 2005).

Expression of the chlamydial murC-ddl *does not restore intracellular phenotypes to a* Δddl*A* Δddl*B*::kan *mutant of* Shigella.

After determining that the *murC-ddl*$_{Ct}$ encoded a functional D-Ala-D-Ala ligase in *Shigella*, we next wanted to determine if expression of *murC-ddl*$_{Ct}$ could restore the cell-to-cell spread of BS775 in a standard *Shigella* plaque assay (Oaks *et al.*, 1985). In this assay, *Shigella* is allowed to infect a confluent monolayer of mouse L2 fibroblast cells. Once inside, the bacteria divide and spread to neighboring cells where they continue to divide and spread. This growth and cell-to-cell spread by *Shigella* cause the invaded cells to lyse. Thus, the ability of intracellular *Shigella* to grow and spread from cell-to-cell is assessed by the formation of a plaque in the fibroblast monolayer. Plaque formation by BS775 expressing *ddlA*$_{Ec}$ was dependent upon the expression of the *E. coli* gene from the *ara* promoter (Table 2.2.1). Furthermore, expression of *ddlA*$_{Ec}$ in BS775 allowed the *Shigella* mutant to form plaques at an efficiency similar to that of the wild-type parent. In contrast, expression of *murC-ddl*$_{Ct}$ was not sufficient to restore the plaquing phenotype to BS775 (Table 2.2.1).

Table 2.2.1. Plaque formation by Δ*ddlA* Δ*ddlB::kan* mutants of *S. flexneri* expressing chlamydial and *E. coli* D-Ala-D-Ala ligase.

Strain	Inducing (+) or repressing (-) conditions *	Inoculum (cfu/mL)	Avg # of plaques/monolayer	Efficiency of plaque formation
2457T	+	1.18×10^3	43	0.036
	-	1.18×10^3	42	0.036
BS775/pBAD38 (*ddlA*$_{Ec}$)	+	1.78×10^3	51	0.029
	-	1.78×10^6	0	$<5.6 \times 10^{-5}$
BS775/pBAD35 (*murC-ddl*$_{Ct}$)	+	3.2×10^6	0	$<3.13 \times 10^{-5}$
	-	N/D	N/D	N/D

* Inducing condition = 0.2% arabinose; repressing condition = 0.2% glucose. N/D = not determined.

Since BS775 could not form plaques when *murC-ddl*$_{Ct}$ was expressed, we needed to address whether this mutant expressing *murC-ddl*$_{Ct}$ could indeed infect HeLa cells. When induced to express *murC-ddl*$_{Ct}$, BS775 invaded HeLa cells at less than 5% of the efficiency of the wild-type and BS775 expressing *ddlA*$_{Ec}$ as assessed using a standard gentamicin protection assay (data not shown). As visualized under the microscope, it was apparent that not only was invasion reduced when BS775 expressed *murC-ddl*$_{Ct}$ but the bacteria also displayed morphological defects (Figure 2.2.2). No "fireworks" were observed from any cell infected with BS775 expressing *murC-ddl*$_{Ct}$. Fireworks form as the bacteria polymerize host actin at one pole of the bacterial cell. This polymerization of actin drives the movement of the *Shigella* from one cell to another. Furthermore, BS775 expressing *murC-ddl*$_{Ct}$ also appeared to have cell division defects as the division zone between two daughter cells in dividing bacteria often appeared to be stretched or elongated as if they were having difficulties in separation (Figure 2.2.2C and D). To confirm that a defect in cell division did indeed exist, the intracellular growth was monitored over time. By 2.5 hours post invasion, nearly all of the intracellular BS775 expressing *murC-ddl*$_{Ct}$ lost viability (Figure 2.2.3).

Expression of the chlamydial murC-ddl *leads to morphological defects when* Shigella *is grown extracellularly.*

The intracellular morphology defects observed when *murC-ddl*$_{Ct}$ was expressed in BS775 led us to ask if the morphology defects were also present when the bacteria were grown extracellularly. After six hours of growth (late log phase) in LB containing arabinose, morphology defects could be noted (Figures 2.2.4C and D). Many bacteria were

Figure 2.2.2. Invasion of HeLa cells by BS775 expressing *E. coli* and *C. trachomatis* D-Ala-D-Ala ligases. Wild-type 2457T (A) and BS775 expressing $ddlA_{Ec}$ (B) or $murC\text{-}ddl_{Ct}$ (C and D) were allowed to invade a semi-confluent (60%) monolayer of HeLa cells. After a 30-minute invasion period, the cells were washed and incubated with gentamycin for 1.5 hours. At the end of the gentamycin incubation, the cells were fixed and stained with Geimsa. Arabinose was present throughout the course of the assay. Arrows denote actin-associated movement or "fireworks". Cell division morphology defects are denoted with diamondheads.

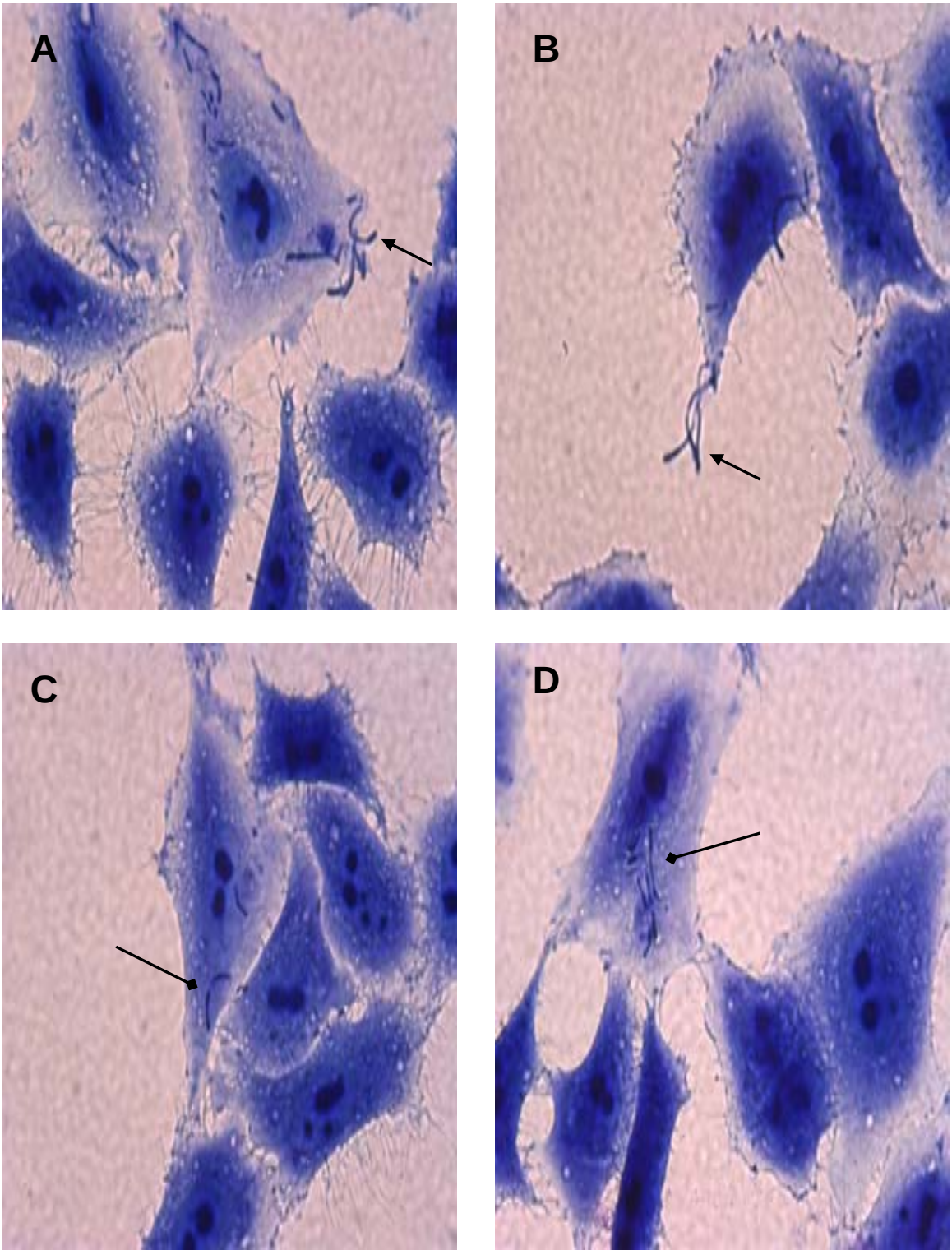

A
B
C
D

Figure 2.2.3. Intracellular viability of BS775 expressing *E. coli* and *C. trachomatis* D-Ala-D-Ala ligases. Wild-type 2457T (square) and BS775 expressing $ddlA_{Ec}$ (circle) or $murC\text{-}ddl_{Ct}$ (triangle) were allowed to invade a semi-confluent (60%) monolayer of HeLa cells. After a 30-minute invasion period, the cells were washed and incubated with gentamycin. At indicated times post-invasion, the HeLa cells were lysed and the released intracellular bacteria were plated onto TSB Congo red agar containing arabinose. The number of bacteria/monolayer was plotted versus time (hr). Arabinose was present throughout the assay.

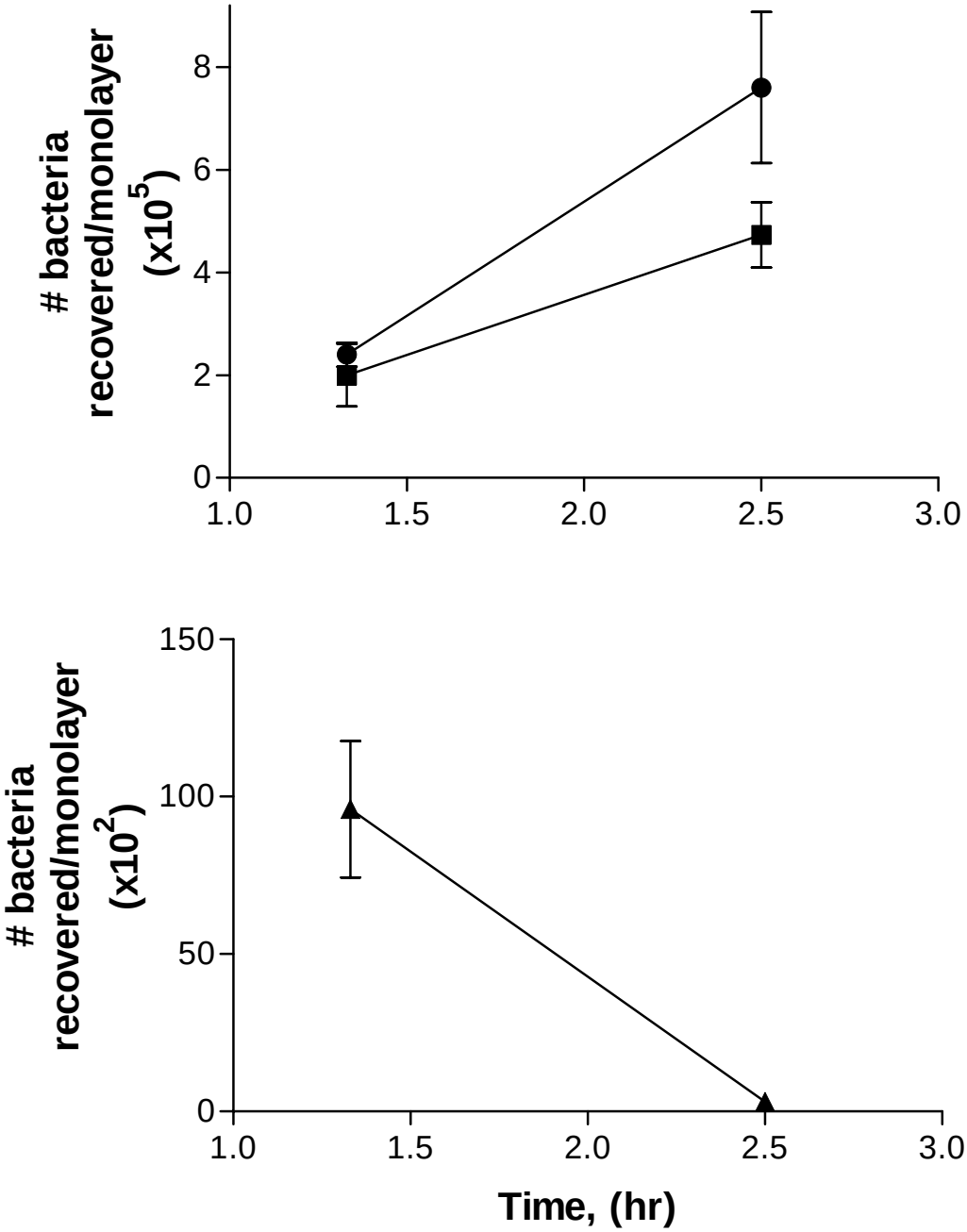
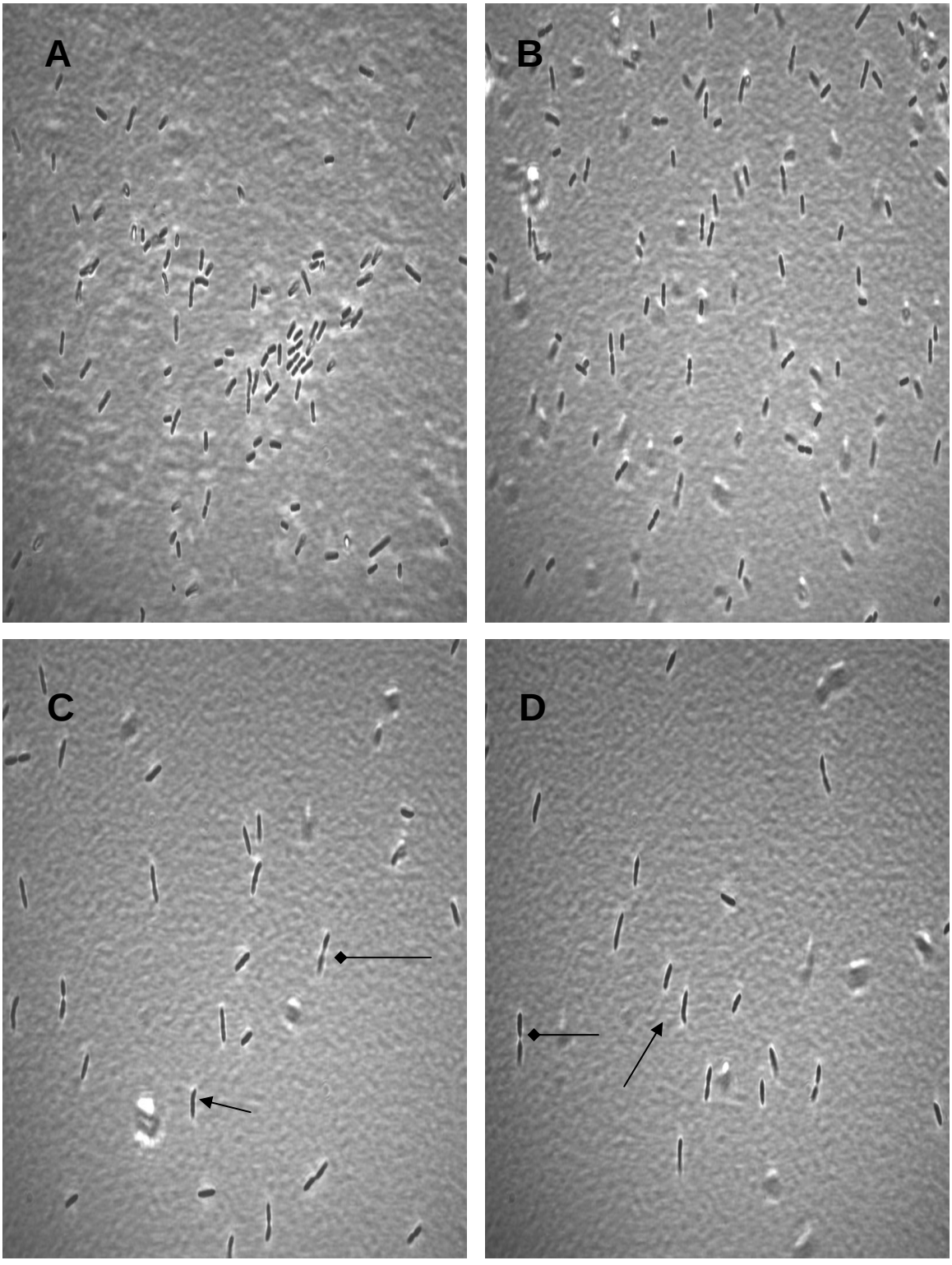

bacteria recovered/monolayer (x10^5)
8
6
4
2
0
1.0
1.5
2.0
2.5
3.0
bacteria recovered/monolayer (x10^2)
150
100
50
0
1.0
1.5
2.0
2.5
3.0
Time, (hr)

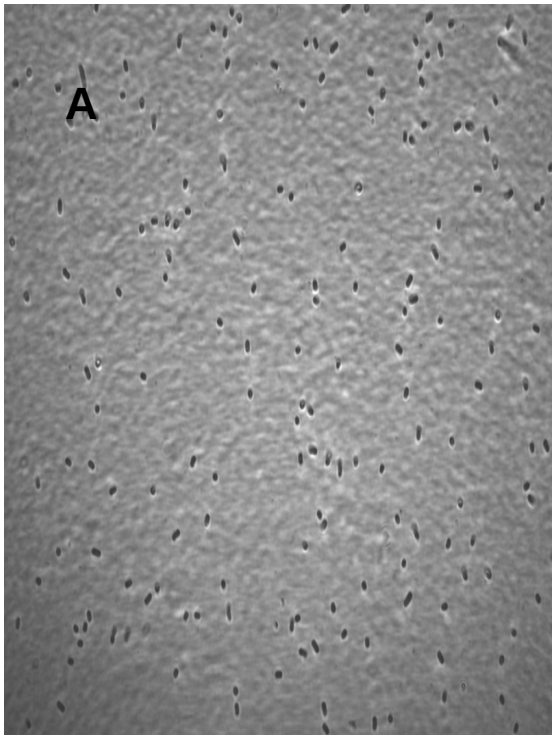
Figure 2.2.4. Morphology of BS775 expressing *E. coli* and *C. trachomatis* D-Ala-D-Ala ligases. Wild-type 2457T (A) and BS775 expressing $ddlA_{Ec}$ (B) or $murC\text{-}ddl_{Ct}$ (C and D) were grown O/N in LB containing arabinose and subcultured into LB containing arabinose. After six hours of growth at 37°C with aeration, the bacteria were visualized by phase contrast microscopy. Arrows denote sickle-shaped bacteria while diamondheads denote defects in separation of daughter cells.

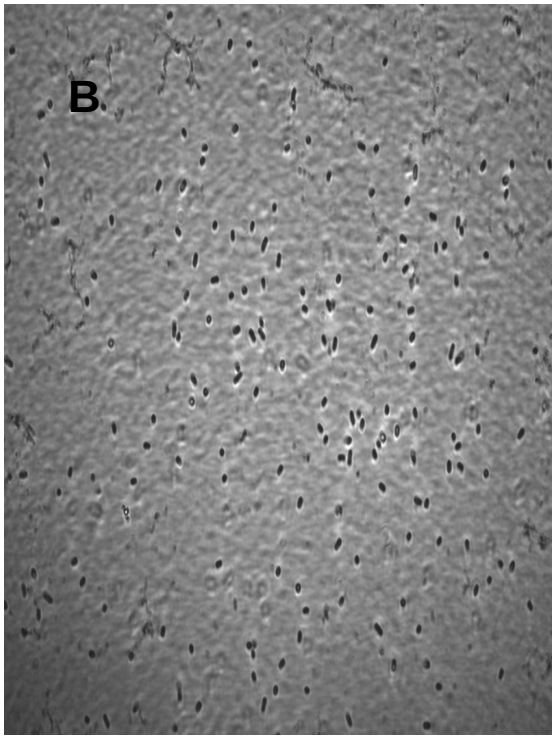

A
B
C
D

sickle-shaped or appeared to have difficulties in separation of the daughter cells during division. After O/N growth (stationary phase) in LB, striking morphological abnormalities were seen when *murC-ddl*$_{Ct}$ was expressed in BS775 (Figure 2.2.5C). The bacteria were significantly larger in size and contained branches and numerous phase-lucent patches. Conversely, no defects were observed in six hour and O/N cultures of BS775 expressing *ddlA*$_{Ec}$ compared to the wild-type *Shigella* parent (Figures 2.2.4A and B and Figures 2.2.5A and B).

Previously, we characterized the chlamydial D-Ala-D-Ala ligase activity of MurC-Ddl using *E. coli* as a heterologous host (McCoy and Maurelli, 2005). During this work, we did not address the issue of morphology phenotypes associated with expression of either *murC-ddl*$_{Ct}$ or *ddlA*$_{Ec}$. Here, when we grew our Δ*ddlA* Δ*ddlB::kan* mutant of *E. coli* expressing *murC-ddl*$_{Ct}$ and *ddlA*$_{Ec}$ O/N in LB medium, no morphology defects were seen in either strain (Figure 2.2.6). This led us to question what differences might exist between the two genera that would give rise to such profound defects in *Shigella*. One difference is the presence of a 220 kb virulence plasmid that encodes for a type III secretion (T3S) system in *Shigella*. T3S systems are needle-like structures that span from the inner cytoplasmic membrane, through the periplasmic space and out though the outer bacterial membrane (Ghosh, 2004). These systems are used to deliver bacterial proteins to eukaryotic cells and are also essential for virulence. We wanted to determine if the cell defects in BS775 expressing *murC-ddl*$_{Ct}$ were linked to the expression and assembly of the T3S system in BS775. In *Shigella* the T3S system is expressed and assembled at 37º but not 30ºC. No difference in morphology was observed when BS775 expressing *murC-ddl*$_{Ct}$ was grown O/N at 30º versus 37ºC (Figure 2.2.7).

Figure 2.2.5. Morphology of BS775 expressing *E. coli* and *C. trachomatis* D-Ala-D-Ala ligases after O/N growth. Wild-type 2457T (A) and BS775 expressing $ddlA_{Ec}$ (B) or $murC\text{-}ddl_{Ct}$ (C) were grown O/N in LB with arabinose and subcultured into LB containing arabinose. After growth O/N at 37°C with aeration, the bacteria were visualized by phase contrast microscopy.


A


B

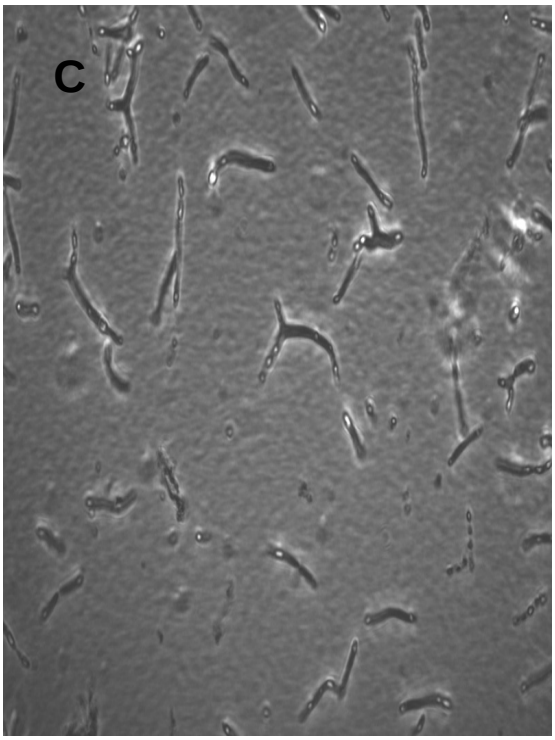

C

Figure 2.2.6. Morphology of ATM718 expressing *E. coli* and *C. trachomatis* D-Ala-D-Ala ligases after O/N growth. ATM718 expressing *ddlA*$_{Ec}$ (A) or *murC-ddl*$_{Ct}$ (B) were grown O/N in LB with arabinose and subcultured into LB containing arabinose. After growth O/N at 37°C with aeration, the bacteria were visualized by phase contrast microscopy.

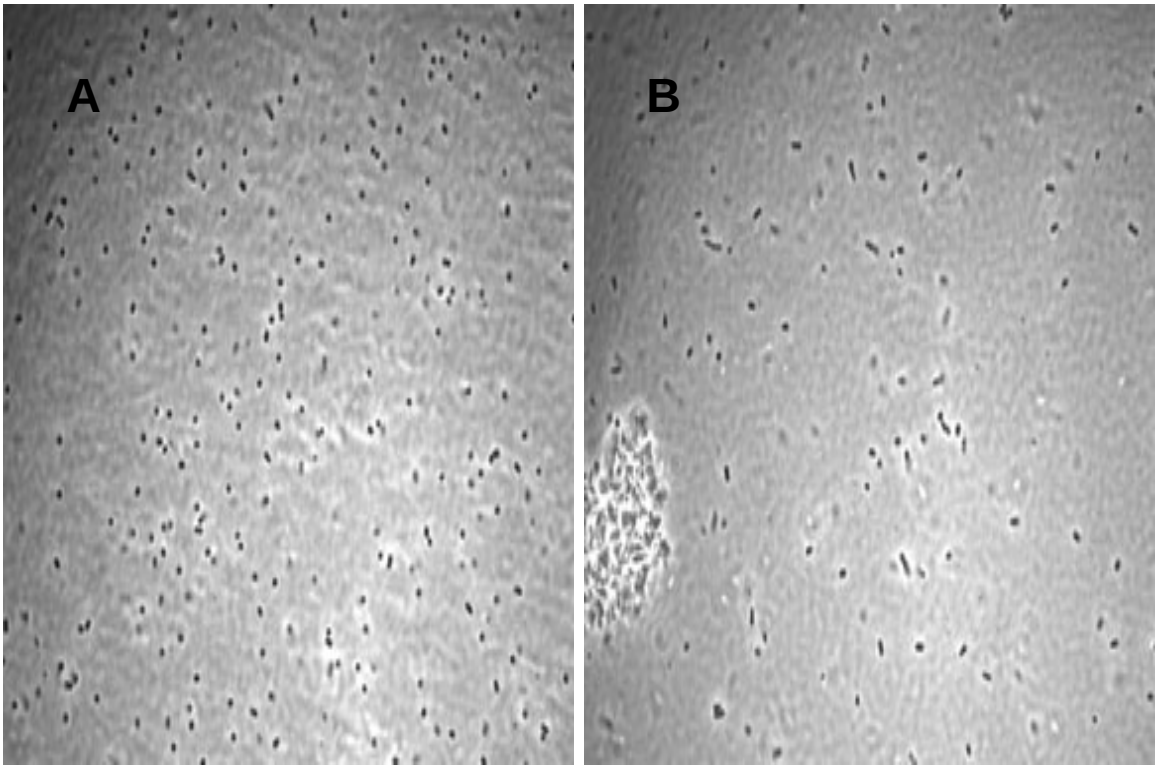
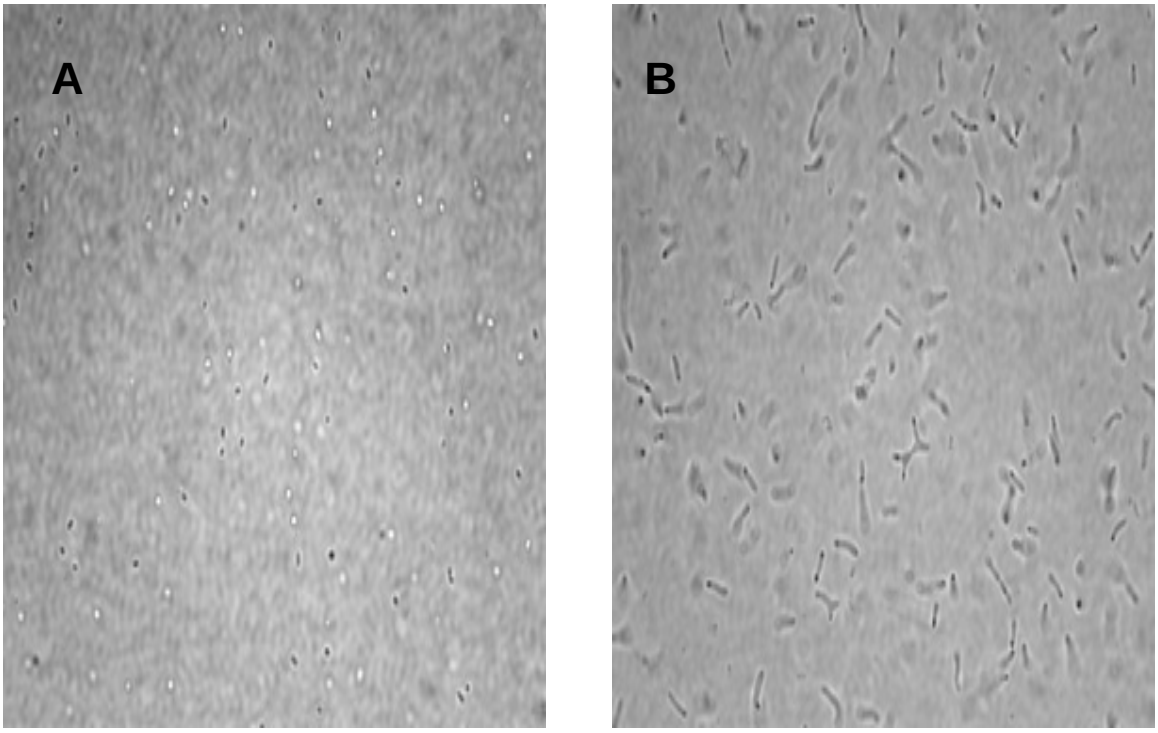

A
B

Figure 2.2.7. Effect of the expression of the *Shigella* T3S system on the morphology of BS775 expressing *E. coli* and *C. trachomatis* D-Ala-D-Ala ligases. BS775 expressing $ddlA_{Ec}$ (A) or $murC\text{-}ddl_{Ct}$ (B) were grown O/N at 30ºC in LB with arabinose and subcultured into LB containing arabinose. After growth O/N at 30°C with aeration, the bacteria were visualized by phase contrast microscopy.


A
B

Another difference between the *E. coli* and *Shigella* heterologous hosts that may be responsible for the morphology defects observed in BS775 expressing *murC-ddl*$_{Ct}$ is the LPS phenotypes of these hosts. LPS is a major constituent of the outer membrane of gram-negative bacteria. The LPS structure consists of lipid A and an inner and an outer core oligosaccharide to which a repeating O antigen region is attached. Bacteria express either smooth LPS, which is composed of O-antigen, complete core oligosaccharides, and the lipid A, or rough LPS which lack O-Ag but possess lipid A and progressively shorter core oligosaccharides. *E. coli* B strains do not express an O-antigen and therefore posses a rough LPS phenotype (Schneider *et al.*, 2002). In contrast, *Shigella* possesses a smooth LPS phenotype. To address whether these morphology defects were related to the presence of the O-antigen in the *Shigella* LPS, we introduced a *galU::tet* mutation into BS775. The LPS of *galU* mutants of *S. flexneri* consists of only the lipid A and inner core region (Sandlin *et al.*, 1995). No difference in morphology was observed when *murC-ddl*$_{Ct}$ was expressed in BS775 Δ*galU::tet* (data not shown).

DISCUSSION

The current lack of a system of genetic manipulation in *Chlamydia* makes it difficult to understand what role a particular enzyme plays in *Chlamydia* biology. Characterization of chlamydial gene products requires that either the expressed protein be purified and characterized biochemically or be expressed in a heterologous host system and characterized by their ability to functionally complement a known mutant phenotype. A small number of chlamydial genes encoding products of predicted function have been characterized by complementation cloning in the heterologous host system of *E. coli* (Hesse *et al.*, 2003; McCoy *et al.*, 2003; McCoy and Maurelli, 2005; Schwoppe *et al.*, 2002; Sweet *et al.*, 2001; Tipples and McClarty, 1995; Zhang *et al.*, 1995). While these studies have been useful in determining the function of these chlamydial enzymes, we questioned whether the facultative intracellular *Shigella*, which is genetically closely related to *E. coli*, would be a better host system for characterization of chlamydial gene products. Therefore, we assessed the functionality of the D-Ala-D-Ala ligase activity of the *C. trachomatis* MurC-Ddl in a D-Ala-D-Ala auxotrophic mutant of *Shigella*. We chose *murC-ddl*$_{Ct}$ because the encoded D-Ala-D-Ala ligase activity has already been characterized in *E. coli* (McCoy and Maurelli, 2005). This allowed us to compare the effects of expressing *murC-ddl*$_{Ct}$ in extracellularly grown *E. coli* and *Shigella* as well as comparing the effects of expression in extracellularly and intracellularly grown *Shigella*.

When we expressed *murC-ddl*$_{Ct}$ in BS775 grown in LB, the viability of the *Shigella* mutant was restored (Figure 2.2.1). These data were not surprising to us based upon previous work in *E. coli* (Chapter 2.1; (McCoy and Maurelli, 2005). One interesting difference between *murC-ddl*$_{Ct}$ complementation in these two heterologous host systems

was the nearly one-log reduction in viable cfu/mL of BS775 when the bacteria reached stationary phase (Figures 2.1.2 and 2.2.1). This difference in viability is likely linked to the morphology defects observed when BS775 expresses *murC-ddl*$_{Ct}$ (Figure 2.2.4, 2.2.5 and 2.2.6). Another key difference between the chlamydial D-Ala-D-Ala ligase activity in *E. coli* and *Shigella* is the apparent stability of the MurC-Ddl$_{Ct}$ in *Shigella*. Under conditions of glucose-repression, the number of viable cfu/mL of BS775 after repression of *murC-ddl*$_{Ct}$ remained constant during the first three hours of growth (Figure 2.2.1B). Under the same conditions, the viability of *E. coli* Δ*ddlA* Δ*ddlB::kan* after repression of *murC-ddl*$_{Ct}$ fell 2-logs after three hours of growth (McCoy *et al.*, 2003).

We believe that the morphology defects seen in BS775 expressing *murC-ddl*$_{Ct}$ correspond to defects in the PG structure. Interestingly, we did not see these same defects when *murC-ddl*$_{Ct}$ was expressed in a Δ*ddlA* Δ*ddlB::kan* mutant of *E. coli* (Figure 2.2.6). These morphology defects are not directly linked to the presence of a T3S system in *Shigella* (Figure 2.2.7). Nonetheless, we believe that these morphology defects affect virulence via the T3S system of BS775. When *murC-ddl*$_{Ct}$ was expressed in BS775, the invasion capacity of this strain was drastically reduced although not completely abolished. One reason for this may be that during early stages of growth, slight defects in the PG structure of BS775 expressing *murC-ddl*$_{Ct}$ may inhibit the proper assembly of the T3S system. In *Salmonella*, defects in PG synthesis have been shown to cause a decrease in the assembly of functional T3S system and a resulting decrease in the delivery of bacterial proteins into eukaryotic cells (Pucciarelli and Garcia-del Portillo, 2003). Of the BS775 that did invade when *murC-ddl*$_{Ct}$ was expressed, the viability was rapidly lost within the HeLa cells (Figure 2.2.3). Upon further examination of these bacteria within the HeLa cells, the

difficulties in separation of two daughter cell during division was more dramatic than what we observed in LB, while non-dividing bacteria appeared swollen (Figure 2.2.2). Likely, the PG morphology defects displayed by BS775 expressing *murC-ddl*$_{Ct}$ before invasion render this mutant more susceptible to host killing mechanisms after invasion.

Similar morphological defects to what we see when we express *murC-ddl*$_{Ct}$ in *Shigella* (*i.e.* sickle-shaped bacteria, difficulties in the separation of two daughter cells, phase-lucent patches and branching) have been described in *E. coli* mutants, specifically in penicillin-binding protein (PBP) mutants. PBPs synthesize and remodel the PG. Recent work has focused on the roles of the low-molecular weight PBPs, PBP4, PBP5, PBP6 and PBP7 in cell division and cell shape determination. PBP5 and PBP6 are monofunctional DD-carboxypeptidases, PBP4 is a bifunctional DD-carboxypeptidase/DD-endopeptidase, and PBP7 is a monofunctional DD-endopeptidase (Popham and Young, 2003; Young, 2001). None of these PBPs is essential for viability of *E. coli.* The sickle-shape, difficulties in separation of daughter cells and the phase-lucent patches are visible in a Δ*pbp1a* Δ*pbp4* Δ*pbp7* Δ*ampH* mutant of *E. coli* that has also been treated with β-lactam antibiotics that specifically inhibit PBP2 and FtsI (PBP3) (Denome *et al.*, 1999). Interestingly, under these conditions the Δ*pbp1a* Δ*pbp4* Δ*pbp7* Δ*ampH* mutants of *E. coli* become coccus-shaped as they reach stationary phase. This defect is in contrast to the branching morphology that we observe in stationary phase when *murC-ddl*$_{Ct}$ is expressed in *Shigella* and is likely due to specific inhibition of PBP2. Inhibition of PBP2 causes rod-shaped *E. coli* to become cocci (Goffin and Ghuysen, 1998; Popham and Young, 2003). Branching phenotypes are observed in PBP5 mutants of *E. coli* that are forced to filament via inhibition of FtsI (PBP3) or FtsZ or that contain deletions of at least two other PBPs

(Nelson and Young, 2001; Nelson, 2000). It has been suggested that the DD-carboxypeptidase activity of PBP5 may regulate the number of cross-links between adjacent peptides that can occur by removing the terminal D-Ala from a pentapeptide chain. This removal of the terminal D-Ala blocks the ability of cross-linking to occur between the resulting quatrapeptide and an adjacent pentapeptide. In the absence of PBP5 alone in *E. coli*, the branching phenotype may be somewhat masked by the presence of the other DD-carboxypeptidases and only uncovered in their absence.

It is surprising that a cytoplasmic protein would give rise to morphologies associated with periplasmic protein mutants of *E. coli*. One way that the expression of MurC-Ddl_{Ct} in *Shigella* could cause such defects is if the Ddl activity has led to an altered PG structure. As discussed in Chapter 2.1, the Ddl activity of MurC-Ddl_{Ct} may possess significant D-Ala-D-Ser activity. If this is the case, both D-Ala-D-Ala and D-Ala-D-Ser dipeptides may be incorporated into the PG-pentapeptide. In *E. coli* and *Shigella*, the DD-carboxypeptidase activity of PBP5 may not be effective in removing a terminal D-Ser in the PG-pentapeptide. Thus, insertion of D-Ala-D-Ser into the PG-pentapeptide would make the bacteria appear to be a PBP5 mutant. In contrast, the removal of the terminal D-Ser may be a function of the DD-carboxypeptidase activity of PBP6, which would compensate for the inability of PBP5 to remove the terminal D-Ser. Surprisingly, there is no PBP6 homologue annotated in the two published *Shigella flexneri* 2a genome sequences (Jin *et al.*, 2002; Wei *et al.*, 2003). Therefore, the presence of D-Ala-D-Ser peptides in the PG of *Shigella*, may allow the bacteria to display the phenotypes of a PBP5 mutant. In support of this possible role of PBP6 is the fact that an *E. coli* PBP6 homologue is the only low-molecular weight PBP encoded in the chlamydial genome. It would be interesting to

see what morphologies an *E. coli* mutant possessing only PBP1a (or PBP1b), PBP2, FtsI (PBP3) and PBP6 would display when expressing *murC-ddl*$_{Ct}$. *Chlamydia* encode for homologues of PBP2, FtsI (PBP3) and PBP6 while either PBP1a or PBP1b is required for the viability of *E. coli.* Likewise, it would be interesting to determine if expression of the *E. coli* PBP6 homologue in *Shigella* has any effect on the bacteria and whether its expression will restore the bacteria to normal morphologies when *murC-ddl*$_{Ct}$ is also expressed.

As has been suggested previously, the ability of chlamydial genes to functionally complement mutations in heterologous host systems may be dependent upon the overall genotype of a particular host (Table 2.2.2) (McCoy *et al.*, 2003). We have speculated that the lack of PBP6 in *Shigella* may be responsible for the morphology and growth differences that exist between our *E. coli* and *Shigella* heterologous host systems, yet we have not tested this hypothesis. We do know that these differences are not related to the expression of a T3S system in *Shigella* or phenotypic differences of the LPS of *E. coli* and *Shigella* (Figure 2.2.7). Based upon the data presented here, we propose that *Shigella* may not be a good heterologous host system for the study of chlamydial PG synthesis genes. However, we cannot rule out the possibility that *Shigella* is a better model system for the characterization of other chlamydial genes. One could envisage that chlamydial gene products that require either host supplied substrates or cofactors may be better analyzed in a *Shigella* background. Thus, the use of *Shigella* as a heterologous host for the characterization of chlamydial gene products may still be of greater benefit than the use of *E. coli* for studying certain aspects of *Chlamydia* biology.

Table 2.2.2. *E. coli* and *Shigella* genetic backgrounds used for characterizing chlamydial PG synthesis genes.

Strain[ϒ]	Genotype	Chlamydial gene	Complementation in a deletion mutant	Expression of chlamydial gene	
				Growth defects*	Morphology defects*
MC4100	F^- *araD*139 Δ(*argF-lac*)U169 *rpsL*150 *relA*1 *deoC*1 *ptsF*25 *rbsR flbB*5301	*murA*	No†	N/A	N/A
C600	F^- *thi*-1 *thr*-1 *leuB*6 *lacY*1 *tonA*21 *supE*44 *rfbD*1 λ^-	*murA*	No‡	N/A	N/A
χ2842	W1485 F^- *rph*-1 λ^- Su^o	*murA*	No‡	N/A	N/A
BL21-CodonPlus	F^- *ompT lon hsd*(rb^- mb^-) dcm^+ Tet^R *gal* λ(DE3) *endA* the [*argU ileY leuW* Cam^R]	*murA*	Yes	Longer lag phase	Phase-lucent bulbs at poles
BL21(DE3)	F^- *dcm ompT lon hsdS gal* λ(DE3)	*murC-ddl*	Yes	No	No
Shigella 2457T	Wild-type *S. flexneri* 2a	*murC-ddl*	Yes	One-log less viable cfu/mL in stationary phase	Sickle-shaped and branched bacteria, difficulty in separation of daughter cells; phase-lucent patches

[ϒ] All strains are *E. coli* unless otherwise noted, * compared to the wild-type parent, N/A = not applicable

† Never obtained Δ*murA* mutant of *E. coli* when $murA_{Ct}$ was expressed, ‡ only obtained gene duplication when $murA_{Ct}$ was expressed

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions.

E. coli strains used in this study are listed in Table 2.2.3. Strains were grown at 37ºC in Luria-Bertani (LB) or M9 minimal medium with aeration or on agar. When appropriate, medium was supplemented with ampicillin (100 μg/ml), kanamycin (50 μg/ml), chloramphenicol (15 μg/ml), arabinose (0.1%), glucose (0.2%) or D-Ala-D-Ala (400 μg/ml). D-Ala-D-Ala and DCS were purchased from Sigma Chemical Co. (St. Louis, MO).

Virulence assays.

Plaque assays were performed with confluent L2 mouse fibroblast monolayers as previously described (Oaks *et al.*, 1985). Briefly, confluent monolayers of L2 mouse fibroblast cells were infected for 90 min at 37°C in 5% CO_2 with either wild-type *S. flexneri* 2a (2457T) or BS775 expressing either $ddlA_{Ec}$ or $murC\text{-}ddl_{Ct.}$. After 90 min, the infection medium was removed. *S. flexneri*-infected cells were incubated for three days at 37°C in 5% CO_2 after the addition of a 5-ml agarose overlay (0.75% low-melting-point agarose) containing Dulbecco's modified Eagle's medium, fetal bovine serum (10%), and gentamicin (20 μg/ml). To visualize plaques at the end of the incubation period, cells were stained with 0.5% neutral red for 2 h at 37°C in 5% CO_2.

Invasion assays were performed in HeLa cells by using the gentamicin protection modification as previously described (Hale and Formal, 1981). Briefly, an overnight culture of bacteria was subcultured into fresh medium containing antibiotics and arabinose (0.2%), and grown to mid-exponential phase. At all subsequent steps, arabinose (0.2%)

Table 2.2.3. Relevant Strains and Plasmids.

Strain or Plasmid	Relevant Genotype	Source or Reference
Shigella		
2457T	Wild-type *S. flexneri* 2a	(Formal *et al.*, 1958)
BS773	2457T Δ*ddlA::kan*	This work
BS774	2457T Δ*ddlA*	This work
BS775	2457T Δ*ddlA* Δ*ddlB::kan*	This work
Plasmids		
pAJM35	pBAD18::*murC-ddl*$_{Ct}$; coding sequence from *C. trachomatis* serovar L2	(McCoy and Maurelli, 2005)
pAJM38	pBAD18::*ddlA*$_{Ec}$; coding sequence from *E. coli* BL21(DE3)	(McCoy and Maurelli, 2005)

was present in all media. The bacteria were washed in DMEM, resuspended in 1.0 mL of DMEM to an OD_{600} of 0.72/mL, and spun onto subconfluent (60%) HeLa cell monolayer in 35-mm dishes at 6,000 rpm for 10 min. After incubation at 37°C for 30 min, cells were washed with warm phosphate-buffered saline (PBS) three times, and the medium was replaced with warm DMEM containing 50 µg/mL of gentamicin. After 30 min incubation at 37°C in the presence of gentamicin, the cells were rinsed and treated as described above. Cells were incubated for an additional hour at 37°C and then rinsed three times with warm PBS. To evaluate the formation of protrusions in the eukaryotic cell membrane caused by intracellular movement of the bacteria, infected cells were fixed with a solution of 0.2% glutaraldehyde-2% formaldehyde in PBS for 5 min at 4°C before staining them with Giemsa (Sandlin *et al.*, 1996).

Intracellular viability assays were performed similar to the invasion assays with some modification. After a 30 minute invasion period, the HeLa cells were washed and incubated with DMEM containing gentamicin. At indicated times post-invasion, the HeLa cells were washed with 1X PBS and lysed in 1 mL of 0.5% triton X-100. Dilutions of the lysed HeLa cells were plated onto TSB Congo red agar containing arabinose in order to determine the number of viable intracellular bacteria.

Chapter 3

STRATEGIES TO IDENTIFY AN ALANINE RACEMASE IN *CHLAMYDIA TRACHOMATIS*

ABSTRACT

The genetic and biochemical characterization of the *Chlamydia* D-Alanyl-D-Alanine (D-Ala-D-Ala) ligase suggests that D-alanine (D-Ala) plays an essential role in *Chlamydia* biology. However, no alanine racemase homologues have been identified in the *Chlamydia* genomes. It has been postulated that *Chlamydia* acquires D-amino acids from the host yet there is no evidence to support the presence of D-amino acids in mammalian cells. Here, we address the issue of the source of D-Ala in *Chlamydia* by constructing Δ*dadX* Δ*alr::kan* double racemase mutants of *Escherichia coli* and *Shigella flexneri* 2a. These mutants require exogenous D-Ala for growth in LB and mammalian cells, respectively. Viability of *E. coli* Δ*dadX* Δ*alr::kan* was not supported in LB medium containing the intracellular milieu of approximately fifty L929 mammalian cells for every one bacterial cell. Screening specific chlamydial genes as well as a *C. trachomatis* serovar L2 genomic library for racemase activity using *E. coli* Δ*dadX* Δ*alr::kan* on LB agar medium failed to identify a candidate chlamydial gene that encodes alanine racemase activity. Likewise, screening specific chlamydial genes and the *C. trachomatis* genomic library using intracellular *S. flexneri* Δ*dadX* Δ*alr::kan* in a plaque assay also failed to identify a chlamydial alanine racemase homologue. Surprisingly, in the absence of

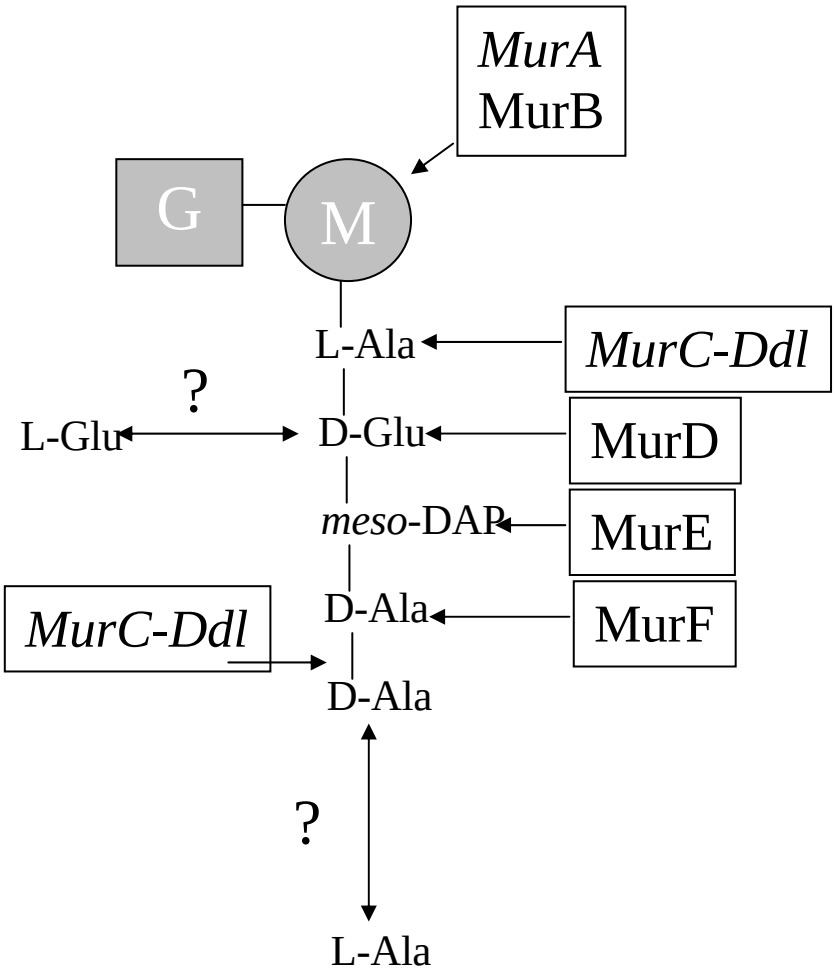
exogenous D-Ala, D-Ala auxotrophy suppressor mutants of both *E. coli* Δ*dadX* Δ*alr::kan* and *Shigella* Δ*dadX* Δ*alr::kan* were obtained. Taken together, our data support the notion that D-Ala is not present in mammalian cells and as a consequence chlamydiae must synthesize their own D-amino acids. However, the pathway of D-Ala synthesis in *Chlamydia* currently remains unknown.

INTRODUCTION

In bacteria, the pentapeptide (Figure 3.1) of peptidoglycan (PG) is unique in the fact that three of the five amino acids that comprise this structure are in the D-configuration (van Heijenoort, 2001). Specifically, D-glutamate (D-Glu) and D-alanine (D-Ala) are essential components of the cell wall and ultimately, required for the viability of the bacteria. In nature, amino acids are found as the L stereoisomer. As a result, bacteria must either obtain D-amino acids from an exogenous source or synthesize D-amino acids from L-amino acids via bacterial racemase enzymes.

The existence of PG in *Chlamydia* has been termed the "chlamydial anomaly" as these bacteria are sensitive to antibiotics that target PG synthesis and encode for a nearly complete pathway for the synthesis of PG despite the lack of biochemical evidence to support its existence (Chopra *et al.*, 1998; Moulder, 1993). One of the aspects that makes the PG-synthesis pathway in *Chlamydia* incomplete is the absence of homologues for both glutamate racemase and alanine racemase. Interestingly, *Chlamydia* encodes a D-Ala-D-Ala ligase (Ddl) and are sensitive to the antibiotic D-cycloserine (DCS) (Lin and Moulder, 1966; McCoy and Maurelli, 2005; Moulder *et al.*, 1963). DCS is a structural analogue of D-Ala that inhibits cell-wall synthesis by targeting alanine racemases and D-Ala-D-Ala ligases (Neuhaus and Hammes, 1981). Recently, we demonstrated that the *C. trachomatis* Ddl homologue is functional in catalyzing the specific ligation of two D-Ala molecules and is inhibited by DCS (McCoy and Maurelli, 2005). These findings suggest that D-Ala plays an essential role in *Chlamydia* biology.

Figure 3.1. PG disaccharide-pentapeptide synthesis in *Chlamydia*. The PG disaccharide-pentapeptide in *Chlamydia* is predicted to be synthesized as shown based upon genome sequencing data and biochemical characterization of key enzymes. All PG synthesis enzymes (boxed) with the exception of the amino acid racemases (?) are encoded in the chlamydial genomes. Biochemically characterized chlamydial PG synthesis enzymes are italicized. G = *N*-acetylglucosamine, M = *N*-acetylmuramic acid.


MurA
MurB
G
M
L-Ala
MurC-Ddl
?
L-Glu
D-Glu
MurD
meso-DAP
MurE
D-Ala
MurF
MurC-Ddl
D-Ala
?
L-Ala

In the absence of an alanine racemase homologue in the *Chlamydia* genomes, it has been postulated that *Chlamydia* obtains D-Ala from their mammalian host (Chopra *et al.*, 1998) (Ghuysen and Goffin, 1999). Despite this speculation, there is no evidence in the literature that mammalian cells contain D-amino acids that could be incorporated into PB. Moreover, an alanine racemase mutant of *Listeria* is unable to grow within macrophages unless exogenous D-Ala is present (Thompson *et al.*, 1998). Here, we present further evidence that mammalian cells lack significant levels of D-Ala by demonstrating that a *Shigella* alanine racemase mutant cannot grow within mammalian cells unless exogenous D-Ala is added to the growth medium. Furthermore, the intracellular contents from mouse L929 fibroblast cells do not contain sufficient D-Ala to support the growth of an *E. coli* alanine racemase mutant. These data predict that *Chlamydia* must synthesize its own D-Ala. However, we report here that we were unsuccessful in identifying a chlamydial gene that encodes for alanine racemase activity. While we were unable to identify a *Chlamydia* alanine racemase, we were able to obtain spontaneous *E. coli* alanine racemase suppressor mutants that could grow on LB agar in the absence of D-Ala. Furthermore, this mutant is hypersensitive to D-cycloserine (DCS). We conclude that a genetic approach may not be the best strategy to identify a chlamydial alanine racemase. However, identifying how *E. coli* alanine racemase suppressor mutants grow in the absence of D-Ala may provide some insight into how *Chlamydia* survives in the absence of racemase homologues. Finally, we suggest ways in which a chlamydial alanine racemase may be uncovered.

RESULTS

Construction of E. coli and Shigella D-Ala racemase activity reporter strains.

D-Ala plays a vital role in peptidoglycan synthesis and thus is essential for the growth of bacteria. Our recent biochemical characterization of the D-Ala-D-Ala ligase activity of the *C. trachomatis* MurC-Ddl fusion protein supports the notion that D-Ala is also vital to the biology of *Chlamydia* (McCoy and Maurelli, 2005). However, in the absence of an apparent racemase in the chlamydial genomes, the source of D-Ala in *Chlamydia* is unknown. Because a system of genetic manipulation is currently not available for *Chlamydia,* we chose to address the question of how *Chlamydia* acquires D-Ala by using the heterologous host systems of both *E. coli* and the facultative intracellular bacteria, *Shigella.*

Although these two genera are very closely related genetically, we have previously noted that *Shigella* may not be the best heterologous host for the study of chlamydial PG synthesis genes. However, *Shigella* may still be a better heterologous host than *E. coli* for the genetic study of chlamydial gene products that utilize host substrates or cofactors. One source of the chlamydial D-Ala that has been hypothesized is the mammalian host. Here, we believe that *Shigella* provides an advantage over *E. coli* as a heterologous host due to its ability to survive and grow within mammalian cells.

In *E. coli* and *Shigella,* two enzymes possess alanine racemase activity (van Heijenoort, 2001). Alr is the biosynthetic alanine racemase, which is constitutively expressed for the purpose of generating D-Ala for PG synthesis. In contrast, DadX is the catabolic alanine racemase, which is inducibly expressed under conditions of high L-Ala and repressed by high glucose concentrations. The D-Ala generated by DadX is ultimately

converted via the D-amino acid oxidase DadA to pyruvate for energy. To create our reporter strains, we employed the system of one-step gene inactivation using PCR products as described in Experimental Procedures to remove both the *alr* and *dadX* genes from the genomes of *E. coli* and *Shigella*. Briefly, we deleted the complete open reading frame (ORF) of either *alr* or *dadX* in *E. coli* strain ATM609 and replaced each with a *kan* cassette that is flanked by FRT sequences. P1L4 lysates were grown on each mutant and used to transduce the mutations into *E. coli* BL21(DE3) and *S. flexneri* 2457T. First, the Δ*dadX::kan* mutation was introduced into each strain. Next, each kanamycin resistant transductant was transformed with pCP20, which expresses a yeast Flp-recombinase. The Flp-recombinase recognizes the FRT sequences flanking the *kan* cassette and removes the cassette, rendering each strain Δ*dadX* and kanamycin sensitive. Finally, the Δ*alr::kan* mutation was introduced by transduction into the Δ*dadX* mutants. The resulting *E. coli* and *Shigella* Δ*dadX* Δ*alr::kan* mutants were designated ATM733 and BS754, respectively.

We next assessed the ability of ATM733 and BS754 to grow in the absence of exogenous D-Ala in LB and intracellularly, respectively. As seen in Figure 3.2, in the absence of D-Ala, the viability of ATM733 drastically decreases within the first hour after subculture and continues until nearly all the cells have lysed. In contrast, in the presence of exogenous D-Ala, growth of ATM733 mirrored the growth of the wild-type *E. coli* BL21(DE3) parent strain in both the presence and absence of D-Ala. Similarly, in the absence of exogenous D-Ala, the viability of BS754 in HeLa cells fell by greater than one-log within the first hour post infection (Figure 3.3). By five hours post infection, the number of viable BS754 was below the level of detection.

Figure 3.2. Viability curves of *E. coli* wild-type and Δ*dadX* Δ*alr::kan*. Wild type *E. coli* ATM340 (squares) and the Δ*dadX* Δ*alr::kan* mutant ATM733 (diamonds) were grown at 37ºC in LB medium with (filled symbols) and without (open symbols) exogenous D-Ala. At indicated times, samples were diluted and plated onto LB agar medium containing exogenous D-Ala. The number of viable bacteria per mL was determined and plotted as the $\log_{10}$ viable cells vs time (hrs).

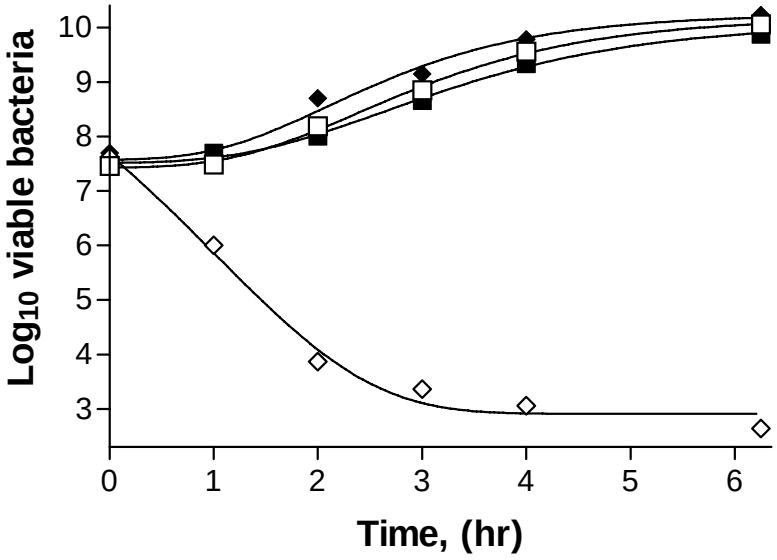
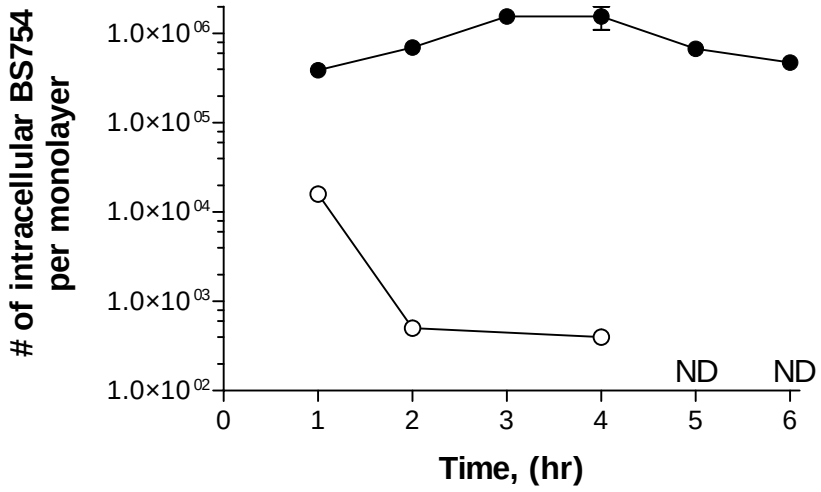

Log10 viable bacteria
10
9
8
7
6
5
4
3
0
1
2
3
4
5
6
Time, (hr)

Figure 3.3. Viability of a Δ*dadX* Δ*alr::kan* mutant of *S. flexneri* 2a in HeLa cells.

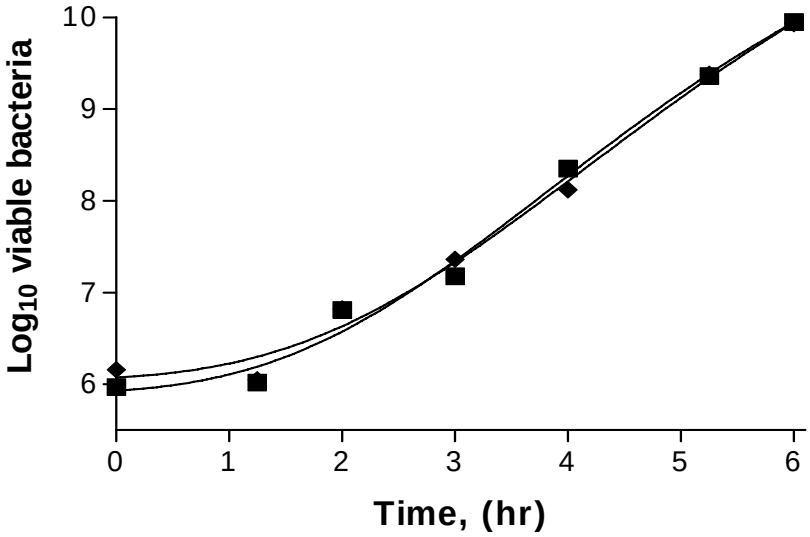
BS754 was grown at 37ºC in TSB medium supplemented with exogenous D-Ala prior to infection. After an initial infection period, BS754 was allowed to continue to grow intracellularly in the presence (filled circles) or absence (open circles) of exogenous D-Ala. At indicated times, the infected HeLa cells were scraped from the dishes and lysed to release intracellular bacteria, which were plated on TSB agar containing exogenous D-Ala. The number of viable bacteria per monolayer was determined and plotted as viable cells vs time (hrs). N/D = none detected

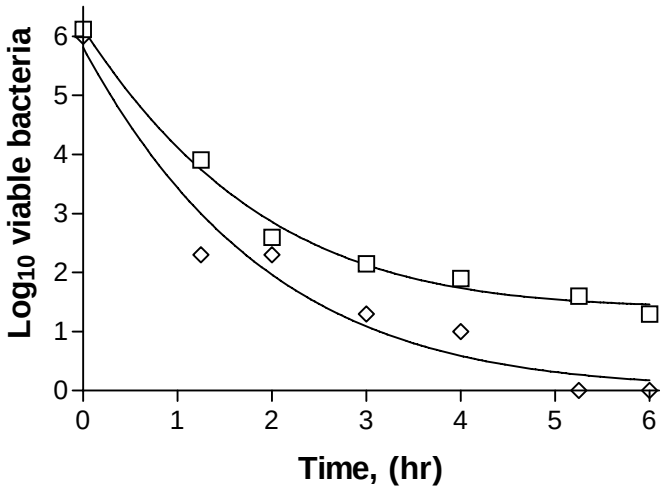

# of intracellular BS754 per monolayer
1.0×10 06
1.0×10 05
1.0×10 04
1.0×10 03
1.0×10 02
ND
ND
0
1
2
3
4
5
6
Time, (hr)

Presence of D-Ala in mammalian cells.

The inability of *Shigella* BS754 (Figure 3.3) and a *Listeria* double racemase mutant (Thompson *et al.*, 1998) to grow intracellularly unless the medium is supplemented with exogenous D-Ala suggests that D-Ala is not present in mammalian cells. However, we wanted to rule out the possibility that there was enough D-Ala in a single mammalian cell to support the morphologically smaller, slower growing *Chlamydia* but not the larger, faster growing *Shigella* or *Listeria.* We reasoned that for the first few hours after subculture sufficient D-Ala may be present at higher mammalian cytosol to bacteria ratios to maintain viability of ATM733 in LB. To assess the validity of this assumption, we suspended mouse L929 fibroblast cells in LB at a density of 5×10^7 cells/mL and released the cytosolic contents into the LB by sonication. After removal of the cell debris by centrifugation, the L929(LB) was filter-sterilized. LB and L929(LB) supplemented with and without D-Ala were inoculated with ATM733. L929(LB) contained the equivalent of the cytoplasmic contents of 50 L929 cells per bacterium inoculated. In the absence of exogenous D-Ala, the viability of ATM733 in LB and L929(LB) was drastically reduced within the first hour of growth (Figure 3.4). By five hours after subculture, no viable cells were detected in the L929(LB) cultures in the absence of exogenous D-Ala. The loss in viability appeared to be specifically related to the D-Ala auxotrophy phenotype as the growth of ATM733 in L929(LB) containing exogenous D-Ala was the same as the growth of ATM733 in LB medium containing exogenous D-Ala.

Figure 3.4. Absence of D-Ala in L929 cells. ATM733 was grown in either LB (squares) or L929(LB) (diamonds) in the presence (filled symbols) or absence (open symbols) of exogenous D-Ala. L929(LB) medium is composed of LB and the cytosolic contents of L929 cells (see Materials and Methods). At indicated times, samples were diluted and plated onto LB agar medium containing exogenous D-Ala. The number of viable bacteria per mL was determined and plotted as the $\log_{10}$ viable cells vs time (hrs).


Log10 viable bacteria
10
9
8
7
6
0
1
2
3
4
5
6
Time, (hr)


Log10 viable bacteria
6
5
4
3
2
1
0
0
1
2
3
4
5
6
Time, (hr)

Specific C. trachomatis candidates for alanine racemase activity.

The absence of an apparent alanine racemase in the *Chlamydia* genomes led us to ask if another annotated gene in *Chlamydia* encoded alanine racemase activity. The Alr and DadX enzymes are members of the PLP-dependent family of enzymes (Jansonius, 1998; Mehta and Christen, 1998). PLP-dependent enzymes fall into four families, termed the α, β, D-Ala aminotransferase and alanine racemase families. Two PLP-dependent enzymes of the α family, GlyA and AspC, are encoded in *Chlamydia*. Interestingly, AspC from *Chlamydia* shares homology to a cystalysin in *Treponema denticola,* which has been shown to possess significant alanine racemase activity in vitro (Bertoldi *et al.*, 2003). Thus, we reasoned that the chlamydial GlyA and AspC enzymes may possess alanine racemase activity. We cloned both the *C. trachomatis* glyA and aspC genes under the control of the ara promoter, transformed them individually or together into ATM733 and determined their ability to restore growth to the D-Ala auxotroph. Neither the expression of the chlamydial *glyA* or *aspC* alone nor together was sufficient to support growth of ATM733 in the absence of exogenous D-Ala (Table 3.1).

We next reasoned that since racemases and epimerases are functionally related, the chlamydial *dapF* homologue, which is predicted to be an L,L-diaminopimelic acid epimerase, may also epimerize L-alanine to D-alanine. To test this possibility, *dapF* from *C. trachomatis* serovar L2 was cloned under the control of the *ara* promoter, transformed into ATM733 and screened for its ability to complement the D-Ala auxotrophy in this mutant. ATM733 harboring this plasmid (pNEA4, pBAD18::$dapF_{Ct}$) was unable to grow on LB agar in the absence of exogenous D-Ala (data not shown).

Table 3.1. Absence of complementation of the D-Ala auxotrophy of ATM733 by specific *C. trachomatis* gene products.

Vector*	Titer (cfu/mL) (-) D-Ala	Titer (cfu/mL) (+) D-Ala	Efficiency of Plating	Complementation
pBAD33	6.6×10^{2}	2.5×10^{9}	2.6×10^{-7}	No
pBAD18	3.5×10^{2}	2.4×10^{9}	1.5×10^{-7}	No
pBAD33/ pBAD18*	1.0×10^{3}	1.8×10^{9}	5.5×10^{-7}	No
pBAD33::*aspC*	4.7×10^{2}	5.9×10^{9}	8.0×10^{-8}	No
pBAD18::*glyA*	1.1×10^{3}	5.2×10^{9}	2.1×10^{-7}	No
pBAD33::*aspC*/pBAD18::*glyA**	8.6×10^{2}	3.0×10^{9}	2.9×10^{-7}	No
No vector	4.2×10^{2}	3.0×10^{9}	1.4×10^{-7}	No

* The origin of replication for pBAD18 and pBAD33 are of different compatibility groups. Efficiency of plating is defined as cfu/mL in the absence of D-Ala per cfu/mL in the presence of D-Ala.

Finally, our last logical choice to test for racemase activity was MurC-Ddl. MurC and Ddl are alanine ligases that require L-Ala and D-Ala as substrates, respectively. Only in the *Chlamydiaceae* family of bacteria are these two enzymes encoded as a fusion protein. Interestingly, the closely related environmental *Chlamydiales, Parachlamydia* and *Simkania*, not only encode separate MurC and Ddl homologues but they also encode an alanine racemase in their genomes. Therefore, we asked whether the unique fusion between MurC and Ddl conferred a third activity upon MurC-Ddl. However, on LB agar in the absence of exogenous D-Ala, expression of *C. trachomatis murC-ddl* was unable to restore viability to ATM733 (data not shown).

Based upon the obligate intracellular niche *Chlamydia* occupies, we rationalized that a chlamydial enzyme possessing alanine racemase activity may require a host-supplied substrate or cofactor. We tested this possibility by expressing the chlamydial *glyA, aspC,* and *murC-ddl* genes in *Shigella* BS754 in the plaque assay. Unfortunately, when expressed in BS754, none of these chlamydial genes could restore plaque formation to the same efficiency as the expression of the *E. coli alr* gene (Table 3.2). Together, these results suggest that the chlamydial GlyA, AspC, MurC-Ddl and DapF enzymes do not possess significant alanine racemase activity to rescue D-Ala auxotrophs of *E. coli* and *Shigella*.

Screening a C. trachomatis serovar L2 genomic DNA library for a gene encoding alanine racemase activity.

Since our logical choices of chlamydial alanine racemase candidates failed to demonstrate activity, we constructed a *C. trachomatis* serovar L2 genomic DNA library, which was used to screen for alanine racemase activity in both ATM733 and BS754 by complementation cloning. This library, named pUCL2(a), contains an average chlamydial

Table 3.2. Inability of specific *C. trachomatis* gene candidates to complement D-Ala auxotrophy in BS754

Vector	(-) D-Ala			(+) D-Ala		
	Input titer of BS754 (cfu)	# plaques	Efficiency of plaque formation	Input titer of BS754 (cfu)	# plaques	Efficiency of plaque formation
No vector	1.42×10^{7}	0	$< 7.0 \times 10^{-8}$	1.42×10^{4}	121	0.85 %
pAV45 (alr_{Ec})*	8.6×10^{3}	73	0.85 %	8.6×10^{3}	73	0.85 %
pAJM35 (*murC-ddl*)	9.0×10^{6}	0	$< 1.1 \times 10^{-7}$	9.0×10^{2}	20	2.0 %
pAJM47 (*aspC*)	1.56×10^{7}	0	$< 6.4 \times 10^{-8}$	1.56×10^{3}	17	1.09 %
pAJM48 (*glyA*)	1.38×10^{7}	0	$< 7.2 \times 10^{-8}$	1.38×10^{3}	16	1.16 %

* The *alr* gene from *E. coli* was cloned and expressed from the *C. trachomatis hsp60* promoter as a control. Efficiency of plaque formation is defined as the # of plaques that form on a monolayer of mouse L2 fibroblast cells of a 35 mm^2 dish per input BS754.

genomic DNA insert of 2.2 kb in pUC19 (ampicillin-resistance) and represents a 2-fold coverage at a 99.9% confidence level for containing the complete *C. trachomatis* serovar L2 genome. Using *E. coli* χ1825, which contains a Δ*bio-asd* mutation that makes this strain a diaminopimelic acid auxotroph on LB agar medium, we were able to isolate plasmids encoding the chlamydial *asd* homologue at a frequency of 9.6×10^{-6}. This frequency suggests that we can screen the pUCL2(a) for complementation with high confidence and minimal effort.

Our first attempt to identify a chlamydial gene encoding alanine racemase activity was performed by transforming pUCL2(a) into ATM733 and selecting for colonies that would grow on LB ampicillin (Amp) agar in the absence of D-Ala. We obtained six ampicillin-resistant colonies on LB agar in the absence of D-Ala (a frequency of 10^{-6}). Restriction endonuclease mapping of the pUCL2(a) plasmids isolated from these six alanine racemase candidates identified four classes of plasmids (data not shown). One class contained vector alone suggesting that these colonies arose due to a suppressor mutation that allowed ATM733 to grow in the absence of D-Ala. The plasmids from the other three candidate classes were retransformed into ATM733 to verify that growth of ATM733 in the absence of D-Ala was due to the chlamydial genomic DNA in the library plasmid. Unfortunately, growth in the absence of D-Ala was not due to any of the plasmids isolated (data not shown). Moreover, sequencing of the inserts from these candidate classes confirmed that one class was comprised of vector alone while one class contained a partial sequence of CT872 (*pmpH*) and another class contained portions of CT766 and 767 (*mia* and a gene encoding a probable biotin synthesis protein). The chlamydial DNA in the fourth class was not identified by sequencing.

In the literature, there is evidence that high levels of D-Ser are present in the brain and a serine racemase has been characterized (Schnell, 2004). Although we previously determined that mammalian cells do not contain D-Ala, we did not address whether D-Ser was present. To determine if *Chlamydia* encodes an enzyme that converts any D-Ser it may acquire from a host cell to D-Ala, we transformed pUCL2(a) into ATM733 and selected for transformants that could grow on LB Amp agar containing 500 μg/mL D-Ser. Five colonies arose (a frequency of 5.3×10^{-7}). Restriction endonuclease mapping revealed that each of the plasmids isolated from the five colonies contained insert DNA and that four classes of inserts were present (data not shown). The inserts of these four classes were identified by sequencing. The inserts from class 1 and class 4 contained CT020 (*lepB*) although the DNA had been cleaved at different Sau3AI restriction sites. The *lepB* gene encodes a signal peptidase. The insert from class 2 contained a fragment of the division/cell wall cluster. This fragment encodes the C-terminus of FtsW, a complete MurG, and the N-terminus of MurC-Ddl. As these enzymes are predicted to be encoded in an operon, none is likely to be expressed in this plasmid. Lastly, the insert in class 3 consisted of the N-terminus of CT252 (*lgt*), a prolipoprotein diacylglyceryl transferase, CT253 and CT254 which both encode *Chlamydia*-specific hypothetical proteins, and the C-terminus of CT255 which encodes a conserved hypothetical protein. The diversity of these inserts suggests that the ability of ATM733 to grow in the absence of D-Ala was due to a spontaneous suppressor mutation rather than the conversion of D-Ser to D-Ala.

We next re-addressed the notion that as an obligate intracellular pathogen, *Chlamydia* may encode an enzyme that requires a mammalian-specific substrate or cofactor to generate D-Ala. We again utilized the facultative intracellular double alanine

racemase mutant of *Shigella*, BS754, to screen the pUCL2(a) library using the *Shigella* plaque assay. As seen previously, this mutant can infect eukaryotic cells in the presence of D-Ala but rapidly dies in the host cell once D-Ala is removed (Figure 3.3). Furthermore, plaque formation by BS754 in the absence of exogenous D-Ala occurs at a frequency < 7.0 x 10^{-8} and expression of a functional alanine racemase *in trans* restores intracellular viability and plaque formation (Table 3.2). BS754 was transformed with pUCL2(a) and transformants were selected on TSB agar containing Congo red, Amp and exogenous D-Ala. Four hundred sixty-six Amp-resistant transformants were scraped from the TSB plates, inoculated into TSB containing Amp and exogenous D-Ala, grown to mid-log phase and assayed in a standard plaque assay. No library candidates should have been isolated as the frequency of isolating plasmids encoding the chlamydial *asd* homologue was determined to be 9.6 x 10^{-6}. However, plaques formed at a frequency between 8.6 x 10^{-7} and 2.6 x 10^{-5} (data not shown). Four plaques were randomly chosen from four separate plaque dishes and bacteria from each of the four plaques was again put through the plaque assay. The efficiency of plaque formation in the absence of exogenous D-Ala ranged between 0.01 and 0.18 % (data not shown). This range was 7.7 to 270-fold lower than plaque formation of the same isolated strain in the presence of D-Ala. Isolation of the pUCL2(a) library plasmids from these plaque forming strains of BS754 and subsequent restriction endonuclease mapping revealed that only two of the four plasmids contained insert DNA. One plasmid contained truncated CT624 (*mviN*) and CT625 (*end4*) while the other plasmid contained an internal fragment of CT870 (*pmpF*) DNA.

Spontaneous suppressors of D-Ala auxotrophy.

The results in the previous section for both ATM733 and BS754 suggest that spontaneous suppressors of the D-Ala auxotrophy can be isolated in a double deletion mutant. This was surprising to us since D-Ala plays an integral role in maintaining the integrity of the PG structure by serving to cross-link glycan chains. However, we reasoned that identifying mutations which allow both *E. coli* and *Shigella* to survive in the absence of an alanine racemase may provide some insight into how *Chlamydia* also survives in the absence of an alanine racemase. For this reason, we isolated spontaneous suppressors of D-Ala auxotrophy in ATM733. These suppressors arose at a frequency of 3.5×10^{-7}. Two mutants (ATM765 and ATM766) were single colony purified in the absence of D-Ala and further characterized.

We first determined the efficiency of ATM765 and ATM766 to grow on LB agar supplemented with and without D-Ala. For both mutants, the plating efficiency ranged from 69-145% (Table 3.3). We next examined these mutants for cell wall defects by testing their ability to grow on LB agar containing 1% sodium dodecyl sulfate (SDS) as well as to grow on MacConkey agar, which contains biles salts. SDS and bile salts are pumped out of bacteria via efflux pumps (Andersen, 2003). Defects within the cell envelope are known to disrupt the activity of these efflux pumps, which leads to sensitivity of the bacteria to these detergents (Eswaran *et al.*, 2004). ATM765 and ATM766 were able to grow on both agar media (data not shown). Finally, we determined the MIC of DCS, Amp and chloramphenicol for both mutants as compared to ATM340, the parent strain of ATM733. DCS and Amp target different steps in cell wall synthesis. DCS inhibits the formation of D-Ala and D-Ala-D-Ala dipeptide. In the absence of a D-Ala

source (*i.e.* deletion of both alanine racemases), DCS should have no effect on these mutants as there is not substrate source for the second target of DCS, the D-Ala-D-Ala ligase. Amp blocks the ability of PG glycan chains to be cross-linked between D-Ala and *meso*-DAP. Suppressor mutants of *meso*-DAP auxotrophy in *Mycobacteria* are known to be hypersensitive to β-lactams (Flores *et al.*, 2005). Chloramphenicol is an inhibitor of protein synthesis and was chosen as a control. As seen in Table 3.3, both ATM765 and ATM766 are hypersensitive to DCS and Amp while no effect was seen on chloramphenicol sensitivity. We also determined whether the hypersensitivity to DCS and Amp displayed by ATM765 and ATM766 could be reversed in the presence of exogenous D-Ala. For both mutants, DCS hypersensitivity could be reversed at D-Ala concentrations between 12.5 and 25 μg/mL. In contrast, the concentration of D-Ala needed to reverse Amp hypersensitivity is greater than 400 μg/mL (Table 3.3).

Table 3.3. MIC of cell-wall targeting antibiotics against D-Ala auxotrophy suppressor mutants.

Strain	Efficiency of Plating (%)*	MIC (μg/mL)			Minimal concentration of D-Ala to reverse growth inhibition at MIC (μg/mL)	
		DCS	Amp	Cat	DCS	Amp
ATM340	N/D	100	25	2.5	N/D	N/D
ATM765	139	12.5	6.25	2.5	12.5	> 400
ATM766	78	12.5	6.25	2.5	25	>400

* % efficiency of plating was calculated as the # viable cfu/mL in the absence of D-ala / # viable cfu/mL in the presence of D-Ala x 100. Values represent the average of two independent platings. MICs and minimal concentrations to reverse growth inhibition were determined by the 2-fold serial broth dilution method. The value determined as the MIC for each antibiotic corresponds to the highest antibiotic concentration at which no growth was visible. The minimal concentration of D-Ala to reverse growth inhibition corresponded to the lowest D-Ala concentration at which growth was visible at the MIC of DCS and Amp. N/D = not determined.

DISCUSSION

The *Chlamydiaceae* family of eubacteria are sensitive to antibiotics that target cell-wall synthesis, synthesize penicillin-binding proteins and encode a nearly complete pathway for the synthesis of PG in the genome (Chopra *et al.*, 1998). Despite these facts, PG has yet to be biochemically detected in these organisms. This contradiction has been termed the "chlamydial anomaly" (Moulder, 1993). Recent genetic and biochemical characterization of key enzymes in the chlamydial PG synthesis pathway (Figure 3.1) have suggested that this pathway is functional in synthesizing PG in *Chlamydia* (Hesse *et al.*, 2003; McCoy *et al.*, 2003; McCoy and Maurelli, 2005).

D-amino acids play a significant role in PG synthesis. Specifically, D-Glu and D-Ala constitute three of the five amino acids of the PG pentapeptide (Figure 3.1). Because amino acids are naturally found in the L-form, bacteria utilize racemases to synthesize D-amino acids from their respective L-isomers. Interestingly, *Chlamydia* does not encode a glutamate racemase or an alanine racemase. The absence of these racemases from the chlamydial genomes may not seem relevant in light of the fact that PG has yet to be biochemically detected in these organisms (Chopra *et al.*, 1998; Moulder, 1993). However, this observation really is significant as it has been known for over forty years that D-Ala can reverse the sensitivity of *Chlamydia* to DCS, a substrate analogue of D-Ala that disrupts PG synthesis by inhibiting the activities of both alanine racemases and D-Ala-D-Ala ligases (Ddl) (Lin and Moulder, 1966; Moulder *et al.*, 1963). Furthermore, recent genetic and biochemical data have shown that one of the activities of the unique *Chlamydia* MurC-Ddl fusion protein is D-Ala-D-Ala ligase activity (McCoy and Maurelli, 2005). *C. trachomatis* MurC-Ddl catalyzes the specific ligation of two D-Ala molecules. Together,

these data demonstrate that D-Ala plays a significant role in the biology of *Chlamydia* and supports the hypothesis that *Chlamydia* must either acquire D-Ala from an exogenous source or synthesize D-Ala from L-Ala.

For many years it has been thought that D-amino acids were exclusively synthesized by bacterial racemases for the synthesis of PG. However, there is now evidence that D-amino acids are synthesized in eukaryotes. A few reports have detected D-amino acids in invertebrate peptides (Kreil, 1997). In these organisms, L-amino acids in biologically inactive peptides are post-translationally modified to a D-amino acid, thereby making the peptide biologically active. The immunosuppressive agent cyclosporin A is synthesized in a non-ribosomal manner by certain fungi. One of the amino acids in cyclosporin A is D-Ala. The source of D-Ala in cyclosporin A is a fungal alanine racemase (Hoffmann *et al.*, 1994). An alanine racemase has also been cloned from the fission yeast, *Schizosaccharomyces pombe* and has been shown to play a major role in D-Ala catabolism (Uo *et al.*, 2001). In the mammalian brain, D-amino acids, specifically D-aspartate (D-Asp) and D-serine (D-Ser), have also been detected at significantly high levels (Schnell, 2004). Their presence in the brain was originally thought to have derived from either a dietary source or from the activity of intestinal bacteria. However, a serine racemase has recently been isolated from mammalian brain tissue.

Based upon the findings of D-amino acids and amino acid racemases in eukaryotes, it has been speculated that the source for D-amino acids in *Chlamydia* is the mammalian host (Chopra *et al.*, 1998; Ghuysen and Goffin, 1999). While this notion is reasonable, several lines of evidence suggest that this is likely not the case. Despite the high concentration of D-Asp and D-Ser in the mammalian brain, D-amino acids have not been

detected in other mammalian cells. Furthermore, *Listeria* (Thompson *et al.*, 1998) and *Shigella* (Figure 3.3) double alanine racemase mutants are not viable in mammalian cells unless exogenous D-Ala is present. Although *Listeria* and *Shigella* double racemase mutants cannot grow in mammalian cells unless exogenous D-Ala is supplied, we speculated that sufficient D-Ala may be present in one mammalian cell to support growth of the morphologically smaller, slower growing *Chlamydia* but not the larger, faster growing *Shigella*. To test this hypothesis, we attempted to grow an *E. coli* double alanine racemase mutant (ATM733) in LB containing the cytosol of fifty mouse L929 fibroblast cells per every ATM733. In this medium, viability of ATM733 was rapidly lost within the first hour. This loss in viability was directly due to the absence of sufficient D-Ala to support growth of the *Shigella* mutant as the presence of exogenous D-Ala restored viability to the mutant in the L929(LB) medium (Figure 3.3). Futhermore, the wild-type *Shigella* parent was able to grow as efficiently in this medium as in LB (data not shown). Together, these data suggest that there is not enough D-Ala to sustain growth of *E. coli* for even one generation (Figure 3.4). Together, our data and the *Listeria* data argue against the mammalian host as the source for D-Ala in *Chlamydia* and supports the notion that *Chlamydia* must synthesize D-Ala by a chlamydial encoded enzyme.

Since we were unsuccessful in genetically detecting alanine racemase activity from our candidate genes, we decided to screen a chlamydial genomic DNA library by complementation cloning. Although we were able to isolate transformants of our *E. coli* double racemase mutant that grew in the absence of exogenous D-Ala on LB agar, the growth phenotype was not due to the chlamydial DNA inserted in the library vector. We next reasoned that, as an obligate intracellular bacteria, *Chlamydia* may encode an enzyme

that can generate D-Ala but requires a host substrate or cofactor. To test this hypothesis, we screened our chlamydial genomic DNA library in a *Shigella* double alanine racemase mutant via a standard *Shigella* plaque assay. Using this assay, we were unable to isolate a plasmid that restored viability to BS754 in the absence of exogenous D-Ala although we were able to isolate spontaneous mutants of BS754 that could form plaques in the absence of exogenous D-Ala.

There are a few possibilities as to why we were unsuccessful in finding a chlamydial gene encoding alanine racemase activity. One reason is that *E. coli* and *Shigella* may be poor choices as heterologous host systems. These bacteria have faster growth rates and are morphologically larger than *Chlamydia*, likely synthesizing a significantly larger concentration of PG per bacterium. A chlamydial racemase may not possess sufficient activity to support the faster growth rate and larger PG structure of *E. coli* and *Shigella*. Another reason is the level of expression of the cloned racemase in these heterologous host systems. Expression of chlamydial genes in the pUCL2(a) library is driven from the native chlamydial promoter. Although we have not experienced problems with expression in *E. coli* of other chlamydial genes from their native promoters, it is possible that the native chlamydial promoter and/or ribosomal binding site of a chlamydial alanine racemase may not be efficiently recognized in *E. coli* and *Shigella.* If this is the case, poor expression of the chlamydial racemase may be the reason for our inability to detect alanine racemase activity by complementation. The codon usage differences between *Chlamydia* and our host systems may also account for why we were unable to detect the expression of a chlamydial alanine racemase in either *E. coli* or *Shigella.* Codon usage differences have not played a significant factor in our ability to sufficiently express

other chlamydial genes in either *E. coli* or *Shigella* so we do not believe this to be the case here (McCoy *et al.*, 2003). Finally, it is possible that two or more unlinked chlamydial genes encode for enzymes that function together to generate D-Ala.

We believe that the most likely reason we were unable to identify a chlamydial gene encoding alanine racemase activity is that the alanine racemase is not efficient enough to be detected by genetic complementation in our heterologous host systems. Despite the fact that AspC and GlyA are known to possess alanine racemase activity, the level of alanine racemase activity displayed by these enzymes is most likely too low to genetically complement D-Ala auxotrophy in *E. coli*. A better approach to identifying a chlamydial enzyme possessing alanine racemase activity may be to first identify a chlamydial enzyme that possess alanine racemase activity *in vitro*. Because *Chlamydia* is an obligate intracellular organism and can obtain amino acids from the host, chlamydial enzymes predicted to be involved in amino acid biosynthesis would make good candidates to overexpress in and purify from our *E. coli* double racemase mutant and test for alanine racemase activity using the *in vitro* assay reported by Bertoldi *et al* (Bertoldi *et al.*, 2003)**.** Both AspC and GlyA, which have alanine racemase activity and are predicted to be involved in amino acid biosynthesis, would be good initial candidates using this approach. Similarly, DapF and MurC-Ddl, which we reasoned may have racemase activity based on their predicted and known activities, respectively, would also be added to our *in vitro* screen. A similar approach would be to PCR amplify and clone all of the *C. trachomatis* ORFs in an expression vector in order to generate a defined expression library in ATM733. Because ATM733 lacks both *dadX* and *alr*, individual lysates over-expressing the chlamydial genes in microtiter plates can be screened for alanine racemase activity. Pooled

lysates from a random expression library is probably not a good approach to identifying an alanine racemase. It is likely that the activity of the chlamydial alanine racemase is so low that it may be masked by the other overexpressed proteins in the pools. For the same reason, separation of proteins from a crude chlamydial lysate using different chromatographic techniques to identify and purify a chlamydial enzyme with alanine racemase activity is also likely not a good approach.

Because D-Ala is crucial to the formation of the PG structure, we were surprised to isolate D-Ala auxotrophy suppressor mutants of both ATM733 and BS754 at such a high frequency (10^{-7} to 10^{-5}). We have begun analyzing two of the *E. coli* D-Ala auxotrophy suppressor mutants (ATM765 and ATM766). These mutants grow as efficiently in the absence of D-Ala as they do in the presence of D-Ala suggesting that they are true suppressor mutants (Table 3.3). To our knowledge, this is the first report of suppressors of D-Ala auxotrophy in a double racemase mutant of *E. coli.*

Surprisingly, the suppressor mutants are hypersensitive to DCS (Table 3.3). DCS targets both alanine racemases and D-Ala-D-Ala ligases. In the genomes of our suppressor mutants, both alanine racemases are deleted thus no D-Ala is being synthesized. Since presumably no D-Ala is being synthesized in these mutants, there is no substrate for the D-Ala-D-Ala ligases. Thus, these suppressors should be resistant to DCS. One way that ATM765 and ATM766 could be hypersensitive to DCS is if either of the *E. coli* D-Ala-D-Ala ligases, DdlA or DdlB, has a mutation that allows other D-amino acids to more efficiently be utilized as substrates. Two reasons argue that this is not the case in our suppressor mutants. First, D-Ala-D-Ala ligases have two D-amino acid binding sites. Mutations would be required in both binding sites in order for a new D-amino acid-D-

amino acid dipeptide to be efficiently synthesized. Two mutations would not be seen at such a high rate of suppression (10^{-7}). Second, the efficient insertion of a dipeptide other than D-Ala-D-Ala into the PG pentapeptide requires that mutations also occur in both MurF, the enzyme that adds D-Ala-D-Ala to the growing PG-pentapeptide chain, and transpeptidating PBPs, which catalyze the linkage between the penultimate D-Ala and an adjacent *meso*-DAP. Again, we would not see these mutations at the high frequency of suppression that we observed for ATM733.

The hypersensitivity to DCS suggests that the suppressor mutation is allowing D-Ala to be synthesized. While we have yet to identify the mutation responsible for this phenotype, we can speculate as to enzymes that may carry the suppressor mutation. One possibility is MurI. MurI is the glutamate racemase, which converts L-Glu to D-Glu. A mutation in this enzyme may alter the substrate specificity such that L-Ala can be racemized to D-Ala. Another possible enzyme is DadA. DadA is a D-amino acid oxidase. The D-Ala generated by the catabolic racemase DadX is subsequently converted to pyruvate by DadA. While this reaction proceeds from D-Ala to pyruvate, a mutation in DadA may allow the reverse reaction to occur more efficiently. We are currently working to identify and characterize the suppressor mutation in ATM765 and ATM766 in hopes that it may provide us with some knowledge of how *Chlamydia* is able to grow in the absence of an alanine racemase in spite of the importance of D-Ala in *Chlamydia* biology. We are also in the process of isolating more D-Ala auxotrophy suppressor mutants in order to determine if the mutation(s) in ATM765 and ATM766 are the only suppressors that can arise.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions.

Strains used in this study are listed in Table 3.4. Strains were grown at 37ºC in either Tryptic Soy Broth (TSB) or Luria-Bertani (LB) with aeration or on TSB or LB agar. When appropriate, medium was supplemented with ampicillin (100 μg/ml; Amp), kanamycin (50 μg/ml; Kan), chloramphenicol (15 μg/ml), arabinose (0.2%), glucose (0.2%), or D-Ala (400 μg/ml). D-Ala and DCS were purchased from Sigma Chemical Co. (St. Louis, MO). SOC medium contains 2% Bactotryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose.

Construction of E. coli and Shigella ΔdadX Δalr::kan.

ATM733 and BS754 (*E. coli* and *Shigella* Δ*dadX* Δ*alr*::*kan*, respectively) were created using the one-step gene inactivation method described by Datsenko and Wanner (Datsenko and Wanner, 2000). Briefly, a *kan* cassette flanked by FRT sequences was PCR amplified such that it lies between 50 nucleotides homologous to the 5' and 3' ends of the DNA coding sequence of *dadX* or *alr*. The amplified DNA was transformed into *E. coli* BW25113 expressing *gam bet* and *exo*, and recombinants were selected on LB agar containing kanamycin. The deletion/insertion event was verified by PCR (see Table 3.4 for all strain numbers). A P1L4 lysate was grown on each recombinant mutant and used to transduce *E. coli* BL21(DE3) and *S. flexneri* 2a 2457T. First, the Δ*dadX::kan* mutation was transduced into *E. coli* and *Shigella* (ATM340 and 2457T, respectively). After verifying Δ*dadX::kan* by PCR, the resulting strains were transformed with pCP20, which expresses a yeast Flp recombinase and facilitates recombination between FRT sequences.

Table 3.4 Bacterial strains and plasmids.

Strains and Plasmids	Relevant Genotype	Source or Reference
E. coli		
BL21 (DE3)	*E. coli* B F^- *ompT hsdS*(r_B^- m_B^-) *dcm* Tet^+ *gal* λ (DE3) *endA* Hte	Stratagene
ATM730	BL21 (DE3) Δ*dadX::kan*	This work
ATM731	BL21 (DE3) Δ*dadX*	This work
ATM733	BL21 (DE3) Δ*dadX* Δ*alr::kan*	This work
ATM765	Spontaneous D-Ala auxotrophy suppressor mutant of ATM733	This work
ATM766	Spontaneous D-Ala auxotrophy suppressor mutant of ATM733	This work
Shigella		
2457T	Wild-type *S. flexneri* 2a	(Formal *et al.*, 1958)
BS776	2457T Δ*dadX::kan*	This work
BS777	2457T Δ*dadX*	This work
BS754	2457T Δ*dadX* Δ*alr::kan*	This work
Plasmids		
pBAD18	Arabinose-inducible expression vector, Amp^R, $pMB1_{ori}$, moderate copy number	(Guzman *et al.*, 1995)
pBAD33	Arabinose-inducible expression vector, Amp^R, $p15A_{ori}$, moderate copy number	(Guzman *et al.*, 1995)
pBAD33::*aspC*$_{Ct}$	Coding sequence from *C. trachomatis* serovar L2	This work
pBAD18::*glyA*$_{Ct}$	Coding sequence from *C. trachomatis* serovar L2	This work
pNEA4	pBAD18::*dapF*$_{Ct}$: coding sequence from *C. trachomatis* serovar L2	This work
pAJM35	pBAD18::*murC-ddl*$_{Ct}$; coding sequence from *C. trachomatis* serovar L2	This work
pAV45	pCRScript::*alr*$_{Ec}$; coding sequence from MC4100	This work
pGEMT::*aspC*$_{Ct}$	Upstream and coding sequence from *C. trachomatis* serovar L2	This work
pGEMT::*glyA*$_{Ct}$	Upstream and coding sequence from *C. trachomatis* serovar L2	This work

This recombination event ultimately excises the *kan* cassette. Next, the Δ*alr::kan* mutation was transduced into the *E. coli* and *Shigella* Δ*dadX* strains and Kan^R transductants were selected on LB agar supplemented with D-Ala. The resulting transductants were screened for D-Ala auxotrophy.

Viability curves of the E. coli ΔdadX Δalr::kan and S. flexneri ΔdadX Δalr::kan mutants in LB and HeLa cells, respectively.

ATM733 was grown overnight (O/N) at 37ºC in LB in the presence of exogenous D-Ala and subcultured into fresh LB with and without exogenous D-Ala. Subcultures were diluted to an OD600 reading of ~0.03 and grown at 37ºC with aeration. At indicated times post-subculture, culture samples were diluted and plated onto LB agar containing D-Ala to obtain viable counts. After incubation, the cfu were counted and the number of cfu/mL was determined. Viability of the cultures was plotted as the Log10 (cfu/ml) versus time (hours).

Intracellular viability of BS754 was determined using a standard gentamicin protection time course assay. BS754 was grown O/N at 37ºC with aeration in TSB supplemented with exogenous D-Ala and subsequently subcultured into fresh TSB containing D-Ala. The subculture was grown to exponential phase. One milliliter of a 0.35 OD600 culture was washed in phosphate-buffered saline (PBS) and resuspended (1 mL) in 1x Dulbecco's minimal essential medium (DMEM) and D-Ala. Semi-confluent (60%) monolayers of HeLa cells in 35 mm2 dishes were washed with pre-warmed DMEM before 0.6 mL of pre-warmed DMEM containing D-Ala and the 1 mL bacterial cultures were added to each dish. Assay dishes were centrifuged at 3,000 rpm for 10 minutes at room

temperature and subsequently incubated for 30 minutes at 37ºC and 5% CO2. The end of this incubation period was marked as t=0. The infection medium was removed and the monolayers were washed five times with pre-warmed 1x PBS containing gentamicin before pre-warmed DMEM containing gentamicin and supplemented with and without D-Ala was added. Infected monolayers were incubated at 37ºC. At indicated times, the infected monolayers were washed five times with 1x PBS containing gentamicin before 1 mL of 0.5% Triton-X supplemented with and without D-Ala was added to lyse open the HeLa cells. After a five-minute incubation at 37ºC, the lysed HeLa cells were scraped off the dish and the contents were collected. Samples were plated onto TSB agar containing Congo red and D-Ala to calculate the number of intracellular bacteria over time.

Presence of D-Ala in mammalian L929 cells.

Mouse L929 fibroblast cells (1 L) were grown in suspension to a density of ~ 5 x 10^5 cells/mL. Cells were centrifuged at 2000 rpm for 20 minutes at room temperature and subsequently washed in PBS. Washed L929 cells were resuspended in LB to a density of ~ 5 x 10^7 cells/mL and sonicated on ice 10-15 times at 20 second pulse intervals. The disrupted cells were centrifuged at 12,000 rpm to remove the cell debris and the supernatant was filter-sterilized using a 0.22 μm filter. O/N cultures of ATM733 (~ 5 x 10^9 cells/mL) were inoculated at a density of approximately 1 x 10^6 cells/mL into LB and L929(LB) with and without D-Ala. At indicated times post inoculation, an aliquot of each sample was diluted in LB and plated onto LB agar containing D-Ala to obtain viable counts. Viability of the cultures was plotted as the Log_{10} (cfu/ml) versus time (hours).

Renografin-purification of C. trachomatis EBs and isolation of genomic DNA.

Confluent mouse L2 fibroblast monolayers in four T-175 cm^2 flasks were infected with *C. trachomatis* serovar L2 at an MOI=1 in 2.5 mL of 1X DMEM. After a 2 hour infection period of rocking at 37ºC, the infection medium was removed and replaced with 20 mL of growth medium containing 1X DMEM, 10% fetal bovine serum, 1X non-essential amino acids, and 1 μg/mL cycloheximide. At 40 hours post infection, the spent growth medium was collected on ice and the infected cells were removed from the flask using glass beads in 1X DMEM (10 mL). The cell suspension was added to the spent growth medium on ice, which was then sonicated twice (20 sec pulse). Sonicated cells were centrifuged at 10,000 rpm for 30 minutes at 4ºC to pellet the *Chlamydia*. The resulting *Chlamydia* pellet was resuspended in 16 mL of SPG (250 mM sucrose, 10 mM sodium phosphate, and 5 mM L-glutamic acid) and sonicated for 2-3 seconds. The sonicated *Chlamydia* suspension was split evenly into two 14 mL ultracentrifuge tubes and 5.4 mL of 30% renografin was layered under the chlamydial suspension using a sterile cannula. The chlamydial suspension/renografin was centrifuged at 24,000 rpm for 40 minutes at 4ºC. The resulting pellet was resuspended in 1.2 mL of SPG and overlayed onto a renografin gradient in a 14 mL Beckman Ultracentrifuge tube that consisted of 3 mL of 40% renografin, 4.9 mL of 44% renografin, and 3.9 mL of 54% renografin. The *Chlamydia*/gradient was centrifuged at 20,000 rpm for 1 hour at 4ºC to separate the chlamydial EBs, which collect as a band at the top of the 54% renografin. The chlamydial EBs were removed using a sterile cannula, diluted in SPG to 14 mL and ultracentrifuged at 20,000 rpm for 1 hour at 4ºC. The renografin-purified EB pellet was resuspended in 1 mL

of SPG and the genomic DNA was extracted using the Qiagen Genomic-Tip 100/G DNA extraction kit (Qiagen).

Cloning of the C. trachomatis glyA, aspC, dapF, and murC-ddl genes.

The complete coding sequence of CT432 (*glyA*), CT390 (*aspC*), CT430 (*dapF*), and CT762 (*murC-ddl*) was PCR amplified from renografin-purified *C. trachomatis* serovar L2 genomic DNA using Platinum Taq polymerase (Invitrogen, Carlsbad, CA). Primer design was based on the genome sequence of *C. trachomatis* serovar D (http://www.stdgen.lanl.gov). PCR products were cloned into pGEMT (Promega**)** and subsequently subcloned into either pBAD18 or pBAD33 under the control of the *ara* promoter. The resulting plasmids are listed in Table 3.4. In the case of *glyA* and *aspC*, pGEMT plasmids were also constructed such that they contained the coding sequence and approximately 50 to 100 nucleotides upstream of the ATG start site. In these plasmids, expression of the *glyA* and *aspC* genes are under the control of the native chlamydial promoters.

Construction of a C. trachomatis serovar L2 genomic DNA library.

Renografin-purified *C. trachomatis* genomic DNA was partially digested with Sau3AI and size fractionated using a 10-40% sucrose gradient. Size fractions between 1.5 and 4 kb were pooled, ligated into BamHI-digested pUC19 and transformed into DH5α. Transformants were selected on LB Amp agar with X-gal for blue-white screening of clones containing inserts. Plasmid DNA was isolated from 78 random white colonies and digested using HindIII and ScaI in order to determine the randomness and average insert

size of chlamydial DNA. Approximately 6000 white colonies from six independent ligations were pooled and the plasmid DNA was isolated to create library pUCL2(a). The empty vector background present in pUCL2a was ~10%. Based upon the average library insert size, 6000 colonies represents a two-fold coverage of the complete *C. trachomatis* genome with 99.9% confidence.

Complementation cloning in E. coli ΔdadX Δalr::kan and S. flexneri ΔdadX Δalr::kan.

To screen for a specific chlamydial gene candidate for alanine racemase activity, plasmids (listed in Table 3.1) or combinations of these plasmids were transformed into ATM733 or BS754 and selected on agar medium containing appropriate antibiotics and exogenous D-Ala. After single colony purifying the transformants twice on the appropriate medium containing D-Ala, one colony was grown overnight in LB containing appropriate antibiotics and D-Ala. To screen for activity on agar, 1.0 mL of overnight culture of ATM733 harboring the different plasmids was washed in buffered saline gelatin (BSG) and serially diluted in BSG. Dilutions were plated onto LB agar with and without D-Ala. Plating efficiencies were determined as the # of cfu/ml in the absence of D-Ala / # of cfu/ml in the presence of D-Ala.

To screen for racemase activity intracellularly, a standard *Shigella* plaque assay was utilized. Overnight cultures of BS754 carrying the different plasmids were subcultured into fresh medium supplemented with D-Ala and grown to mid-exponential phase. The cultures were normalized to an OD_{600} reading of 0.3 and 1 mL was washed three times in pre-warmed PBS before the bacteria were allowed to infect confluent monolayers of mouse L2 fibroblast cells in the presence of D-Ala. After a two hour infection period, the infection

medium was removed and the infected L2 cells were overlayed with an agarose medium containing nutrients to support growth of the eukaryotic cells and antibiotics to kill extracellular bacteria and support maintenance of the plasmids, and supplemented with and without D-Ala. After three days at 37ºC and 5% CO_2, the cells were stained with neutral red (0.5%) for two hours at 37ºC prior to removal of the agarose overlay in order to visualize plaque formation. The ability of the chlamydial DNA to complement the alanine racemase defect was assessed by the ability of the *Shigella* racemase mutant to form plaques in the absence of D-Ala compared to their ability in the presence of D-Ala.

In order to screen a *C. trachomatis* library for an alanine racemase candidate, the pUCL2(a) genomic library was transformed into ATM733 and BS754. After one hour of recovery in SOC medium, an aliquot of the transformation was diluted and plated onto agar containing Amp and D-Ala in order to calculate the total number of transformants. For screening the library for growth on agar using ATM733, the remaining transformants were pelleted by centrifugation and plated directly onto LB Amp agar without D-Ala. Transformants that grew in the absence of D-Ala were single colony purified twice on LB Amp agar medium before plasmid DNA was harvested and the insert DNA was sequenced. To screen the pUCL2(a) library by intracellular complementation, BS754 transformants were selected on TSB Amp agar supplemented with D-Ala and subsequently scraped from the agar medium, inoculated into LB Amp with D-Ala, and grown until the culture reached an OD_{600} reading of ~ 0.5. The cultures were then screened through a standard *Shigella* plaque assay without exogenous D-Ala as described in *Experimental Procedures* of Chapter 2.2. Plaques that arose after three days of incubation were picked and plaque

purified. Plasmid DNA was isolated from the plaque forming strains of BS754 and the inserts were sequenced.

Isolation of spontaneous ATM733 D-Ala suppressor mutants.

ATM733 was grown O/N in LB broth containing exogenous D-Ala. One milliliter of O/N culture was pelleted by centrifugation and plated onto LB agar medium without D-Ala. Plates were incubated O/N at 37ºC. Two mutants (ATM765 and ATM766) were colony purified and stocked for future analysis.

Plating efficiency of suppressor mutants of E. coli D-Ala auxotrophy.

O/N cultures of ATM765 and ATM766 in LB were subcultured and grown to mid-exponential phase at 37ºC with aeration. Samples of the culture were diluted in BSG and plated onto LB agar with and without D-Ala. After O/N incubation at 37ºC, the number of viable counts was calculated. Percent plating efficiency equals the # viable cfu/mL in the absence of D-Ala / # viable cfu/mL in the presence of D-Ala.

Determination of minimal inhibitory concentrations (MIC).

ATM340 (wild-type *E. coli* BL21(DE3)), ATM765 and ATM766 were grown O/N in LB at 37ºC with aeration and subcultured into fresh LB. Subcultures were grown to mid-log phase and all OD_{600} values were normalized to 0.5 (~ 2 x 10^8 cfu/mL). LB containing 2-fold serial dilutions of DCS, Amp or chloramphenicol was inoculated with approximately 10^5 cfu/mL. Cultures were grown O/N at 37ºC without aeration. The MIC was determined as the concentration at which bacterial growth was not visible.

To determine the ability of D-Ala to reverse antibiotic hypersensitivity, O/N cultures of ATM765 and ATM766 grown in LB at 37ºC were subcultured and grown to mid-exponential phase. OD_{600} values were normalized to 0.5 (~2 x 10^8 cfu/mL). LB containing either DCS or Amp at the MIC and 2-fold serial dilutions of D-Ala was inoculated with ~10^5 cfu/mL. Cultures were grown O/N at 37ºC without aeration. The minimal concentration of D-Ala that could reverse the growth inhibition of the antibiotic was determined as the concentration at which bacterial growth was visible.

D i s c u s s i o n

Functionality of key PG synthesis enzymes in *Chlamydiaceae.*

When discussing the cell wall of bacteria, it has been generally accepted that nearly all eubacteria synthesize PG with the exception of *Mycoplasma, Pirellula, Ehrlichia,* and *Chlamydia.* Interestingly, with the exception of *Chlamydia,* the genome sequencing data for all of these organisms confirms this notion as few if any PG synthesis enzymes are found to be encoded. In contrast, the *Chlamydia* genomes encode a nearly complete pathway for the synthesis of PG. A general look at many of the chlamydial PG synthesis gene products suggests that these enzymes are functional as critical and invariant amino acids identified in PG homologues of other bacteria have been maintained in the chlamydial enzymes. To address the functionality of the chlamydial PG synthesis pathway, genetic and biochemical characterization of key enzymes in the pathway were undertaken.

Here, the *C. trachomatis* MurA enzyme was chosen to be the first enzyme characterized for two reasons. First, the numerous attempts to detect PG synthesis in *Chlamydia* have specifically attempted to detect MurNAc or MurNAc derivatives without success (Chopra *et al.,* 1998). The inability to detect these compounds has led to the speculation that an amine sugar other than MurNAc may be present in a chlamydial PG structure. Second, the addition of enolpyruvate from PEP to UDP-GlcNAc by MurA is the first step in PG synthesis and commits UDP-GlcNAc to the synthesis of PG. Furthermore, the resulting product of the MurA reaction is not used as a substrate in any other bacterial pathway except the synthesis of MurNAc. The chlamydial MurA does indeed catalyze the addition of enolpyruvate to GlcNAc *in vitro* while *in vivo* expression of *C. trachomatis*

murA in a conditional lethal *E. coli* Δ*murA* mutant restores viability to the distantly related *E. coli* mutant (Figure 1.3 and 1.5) (McCoy *et al.*, 2003).

The active site Cys115 (*E. coli* numbering) has been replaced with Asp in the chlamydial MurA sequence. This same change has been identified in many other organisms (Table D.1). Despite the change, the activity of *Chlamydia* and *Mycobacterium* MurA is maintained although kinetic properties such as the pH optimum are altered (Figure 1.5) (De Smet *et al.*, 1999; McCoy *et al.*, 2003). Likewise, an *E. coli* Cys115Asp MurA mutant is also functional and exhibits kinetic properties similar to the *Chlamydia* and *Mycobacterium* MurA (Kim *et al.*, 1996). This Cys115Asp alteration has been identified as a mechanism of resistance to FOS, a structural analogue of PEP. Thus, organisms containing a Cys115Asp substitution listed in Table D.1 would be predicted to be resistant to FOS. As expected, experiments show that mycobacteria and *Chlamydia* are innately resistant to FOS (De Smet *et al.*, 1999; Kim *et al.*, 1996; McCoy *et al.*, 2003). Since FOS has never been used clinically to treat chlamydial infections, it is not likely that the Cys115Asp substitution is a response to the selective pressure of the antimicrobial therapy. Interestingly, the environmental *Chlamydiale* families, *Parachlamydia* and *Simkania*, encode for a Cys in the MurA active site and would be predicted to be sensitive to FOS (Horn *et al.*, 2004)(Garry Myers, personal communication). It is unknown how or why this change has occurred in the *Chlamydiaceae* family during its evolution. Like *Parachlamydia* and *Simkania* other obligate intracellular bacteria contain a Cys in the MurA active site (Table D.1). Thus the selective pressure in *Chlamydia* was not its strict residence in a mammalian host nor their evolution to animal pathogens as *Parachlamydia* and *Simkania* cause respiratory infections in humans and animals

Table D.1. Alignments around the binding site for fosfomycin in the *murA* gene product (C115, *E. coli* numbering)

		115	
Escherichia coli **S**	111	LPGG**C**TIGAR	120
Bordetella pertussis		LPGG**C**AIGQR	
Brucella suis 1330		LPGG**C**AIGTR	
Coxiella burnetii RSA 493		LPGG**C**AIGSR	
Listeria monocytogenes		LPGG**C**YLGPR	
Listeria innocua		LPGG**C**AIGSR	
Neisseria gonorrhoeae		LPGG**C**AIGSR	
Parachlamydia sp. UWE25		TAGG**C**PIGQR	
Rickettsia prowazekii Madrid E		LPGG**C**AIGAR	
Shigella flexneri 2a **S**		LPGG**C**TIGAR	
Simkania†		LPGG**C**TIGAR	
Streptomyces avermitilis MA-4680		YAGG**C**DLGTR	(MurA1)
		GLGG**C**DIGGR	(MurA2)
Streptomyces coelicolor A3		GLGG**C**DIGGR	
		YAGG**C**DLGTR	
Wigglesworthia glossinidia		LPGG**C**EIGNR	
Wolbachia		FPGG**C**NIGKR	
Yersinia pestis		LPGG**C**AIGAR	
Chlamydia caviae		CVGG**D**AIGER	
*Chlamydia trachomatis**R**		ILGG**D**AIGPR	
Chlamydia muridarum		ILGG**D**AIGPR	
Chlamydia pneumoniae#		TVGG**D**AIGER	
Chlamydia abortus		CVGG**D**AIGER	
Chlamydia psittaci†**R**		CVGG**D**AIGER	
Chlamydia pecorum†		ILGG**D**AIGRM	
Borrelia burgdorferi		LPGG**D**VIGKR	
Corynebacterium diphtheriae		LPGG**D**AIGSR	
Mycobacterium tuberculosis CSU#93 **R**		LPGG**D**AIGSR	
Porphyromonas gingivalis W83		KPGG**D**KIGRR	
Treponema denticola		PPGG**D**VIGRR	
Mycoplasma spp.	no *murA* gene and no peptidoglycan		
Pirellula sp.	no *murA* gene and no peptidoglycan		
Ehrlichia ruminantium	no *murA* gene and no peptidoglycan		

† Garry Myers, personal communication
* Sequence for both *C. trachomatis* serovar D and serovar L2
# Sequence for three sequenced *C. pneumoniae* strains
S Sensitive to FOS (Kim *et al.*, 1996), (McCoy and Maurelli, unpublished)
R Resistant to FOS (De Smet *et al.*, 1999; McCoy *et al.*, 2003), (McCoy and Maurelli, unpublished)

(Corsaro and Venditti, 2004). Clearly, modification of the active site of MurA must impart some selective advantage on the pathogenic *Chlamydia* in their developmental cycle within the mammalian host. It remains to be determined what that advantage is. One possibility is to regulate the activity of the enzyme so as to not deplete the pool of GlcNAc, which can be utilized in other pathways including glycolysis.

Subsequent to the characterization of the *C. trachomatis* MurA, the characterization of the MurC domain of the *C. trachomatis* MurC-Ddl fusion protein was published. At the same time, the work presented here for the characterization of the D-Ala-D-Ala ligase activity of this unique fusion protein was being performed. Together, it was determined that the expression of AA 1-457 of MurC-Ddl or the full fusion protein in an *E. coli* temperature-sensitive *murC* mutant is sufficient to restore growth to the heterologous host at the non-permissive temperature (Figure 2.1.4)(Hesse *et al.*, 2003). Not surprisingly, expression of *C. trachomatis* MurC-Ddl containing alanine replacements of either the invariant K115 or E157 in an *E. coli murC*(ts) mutant is unable to restore viability to the *E. coli* mutant (Figure 2.1.4). This result demonstrates that these invariant residues play a crucial role in the activity of MurC and further attests to the potential of MurC to participate in PG synthesis in *Chlamydia*. In nearly all bacteria, MurC selectively ligates L-Ala to MurNAc *in vivo* yet *in vitro* kinetics suggest that *Chlamydia* MurC may ligate either L-Ala or L-Ser to MurNAc (Hesse *et al.*, 2003). A similar report has been made for the *E. coli* and *Staphylococcus* MurC enzymes (van Heijenoort, 2001). While we cannot exclude the possibility that chlamydial MurC ligates L-Ser to MurNAc *in vivo*, until PG is detected in *Chlamydia*, we assume, based on complementation in the distantly related *E. coli*, that the chlamydial MurC is specific for L-Ala *in vivo*.

The functionality of both the chlamydial MurA and MurC enzymes lend credence to the notion that PG synthesis is occurring in *Chlamydia*. However, the question of the presence of D-amino acids in PG still remained as no amino acid racemases are encoded in the chlamydial genomes despite the presence of a D-Ala-D-Ala ligase. We chose to address this question next. The D-Ala-D-Ala ligase enzyme, Ddl, is encoded as a unique, *Chlamydia*-specific fusion with MurC. One could argue that the unique fusion of the *murC* and *ddl* genes has occurred in chlamydiae to allow the incorporation of L-amino acids rather than D-amino acids into the chlamydial PG peptide as both gene products utilize alanine but in different enantiomer forms. However, expression of *C. trachomatis murC-ddl* in a D-Ala-D-Ala auxotrophic *E. coli* Δ*ddlA* Δ*ddlB* mutant restores viability to the bacteria in the absence of exogenous D-Ala-D-Ala (Figure 2.1.2) (McCoy and Maurelli, 2005). Complementation by the chlamydial Ddl requires the structure of the full fusion protein but not the enzymatic activity of the MurC domain (Figure 2.1.4) (McCoy and Maurelli, 2005). Furthermore, the D-Ala-D-Ala ligase activity is highly specific for D-Ala as a substrate (Figure 2.1.5, Table 2.1.1) (McCoy and Maurelli, 2005). This result is in contrast to the MurC activity as a UDP-muramic acid:amino acid ligase as discussed above, which is independent of the Ddl domain. However, the fact that the Ddl domain of the MurC-Ddl fusion protein is functional as a D-Ala-D-Ala ligase strongly suggests that *Chlamydia* synthesizes a PG-peptide that contains D-amino acids, specifically D-Ala. This conclusion is further supported by the observation that *Chlamydia* spp. are sensitive to the antibiotic DCS, a competitive inhibitor of Ddl. Inhibition of the Ddl activity of MurC-Ddl by DCS can be reversed in the presence of exogenous D-Ala but not L-Ala in *Chlamydia* (Table 2.1.2)(McCoy and Maurelli, 2005) (Moulder *et al.*, 1963).

The functionality of the Ddl domain of the chlamydial MurC-Ddl and the essentiality of D-Ala for chlamydial growth and development brings into question the source of D-Ala in *Chlamydia* as neither an alanine racemase nor a D-alanine aminotransferase was identified in the genome. Bacteria can only obtain D-amino acids by an exogenous source or synthesis by their own means. The fact that *Chlamydia* grows in tissue culture in sterile tissue culture medium suggests that D-amino acids from other bacteria is not the source of chlamydial D-amino acids. But what about the host cell? It has been speculated that *Chlamydia* obtains D-amino acids from the host cell yet two key pieces of evidence contradict this notion (Chopra *et al.*, 1998). First, alanine racemase mutants of *Listeria* and *Shigella* cannot grow inside mammalian cells unless exogenous D-Ala is added to the growth medium (Figure 2.2.1) (Thompson *et al.*, 1998). Furthermore, an alanine racemase mutant of *Francisella* is not viable in mammalian macrophages (Gray *et al.*, 2002). Second, while high levels of D-Asp and D-Ser as well as a serine racemase have been detected in the brain, there is little evidence to support the presence of other D-amino acids in mammalian cells (Schnell, 2004). Of the data in support of D-amino acids in lower eukaryotic cells, the mechanism by which this occurs appears to be post-translational modification of either L-Ala or L-Ser in peptides (Kreil, 1997). Because the Ddl activity of MurC-Ddl is selective for D-Ala and neither L-Ala nor L-Ser can reverse DCS inhibition, post-synthesis modification of the PG peptide is likely not the mechanism by which *Chlamydia* incorporates D-amino acids into PG (Table 2.1.2). It will be intriguing to see how *Chlamydia* synthesizes D-amino acids as it will be a novel pathway.

Other key chlamydial PG synthesis enzymes worthy of characterization.

meso-*DAP biosynthetic pathway*

In PG, *meso*-DAP plays a key role by serving to cross-link adjacent PG peptide units. This cross-linking connects the linear PG glycan strands and provides strength and rigidity to the PG structure. Besides functioning to cross-link glycan strands in PG, *meso*-DAP containing muropeptides also possess biological activities (Boneca, 2005; Johannsen, 1993). Muramyl-tripeptide (MurNAc-tripeptide) from *Bordetella pertussis* and *Neisseria gonorrhoeae* display potent cytotoxic effects on ciliated cells. DAP-containing muropeptides are also recognized by the intracellular pattern recognition molecule, Nod1 (Inohara *et al.*, 2005). Recognition of *meso*-DAP containing muropeptides by Nod1 leads to the activation of NF-κB, which leads to the expression of proinflammatory cytokines, chemokines and growth factors. Recently, Nod1 was shown to detect *C. pneumoniae* in endothelial cells suggesting that *meso*-DAP and PG are present in *Chlamydia.*

Like the PG synthesis pathway, the *meso*-DAP biosynthetic pathway is incomplete in *Chlamydia* (Figure I.3). Homologues of the genes for DapA, DapB and DapF are present in the genomes of the sequenced *Chlamydia* while the genes encoding DapC, DapD and DapE (the succinylase branch) appear to be missing. The preservation of conserved amino acids within the Dap enzyme sequences suggests that the enzymes are functional. For example, Lys161 of *E. coli* DapA has been identified as the active site residue (Cox *et al.*, 2000). Alignment of DapA from the sequenced *Chlamydia* to the *E. coli* DapA reveals that this lysine is conserved throughout the *Chlamydiaceae* family. In *E. coli* DapB, His160 participates in substrate binding while His159 and Lys163 participate in the catalytic function of the enzyme (Cox *et al.*, 2000). These residues are conserved within

the chlamydial DapB sequences as well. Finally, the *Haemophilus influenzae* DapF enzyme contains two conserved cysteines, Cys73 and Cys217, which form a disulfide linkage. Site-directed mutagenesis of both conserved cysteines demonstrate that these amino acids are required for epimerase activity (Cox *et al.*, 2000; Hutton *et al.*, 2003). Both Cys73 and Cys217 are conserved in the DapF sequences of *Chlamydia*. If these enzymes are functional, then *Chlamydia* is able to synthesize tetrahydrodipicolinate (THDP) and epimerize L,L-DAP to *meso*-DAP (Figure I.3). Yet, how is THDP converted into L,L-DAP in the absence of DapC, DapD and DapE? The dehydrogenase pathway bypasses the need for DapC-DapF by directly converting THDP to *meso*-DAP by Dap dehydrogenase. If *Chlamydia* utilizes the dehydrogenase pathway, there would be no need for these organisms to maintain a functional DapF homologue. Interestingly, the *meso*-DAP/lysine biosynthesis pathway in plants contains the same holes as the *Chlamydia meso*-DAP pathway (Hudson *et al.*, 2005). How plants synthesize *meso*-DAP in the absence of the succinylase pathway has yet to be resolved however it has been suggested that direct transamination of THDP to LL-DAP may occur (Cox *et al.*, 2000). The *meso*-DAP synthesis pathway in *Chlamydia* may function analogously to the *meso*-DAP synthesis pathway in plants. Because of the potential novel nature of a THDP to L,L-DAP synthesis in *Chlamydia* that may cross kingdom lines, this pathway deserves study.

PBPs

Since 1947 numerous studies have documented the effects of penicillin on chlamydial growth and development (Barbour *et al.*, 1982; Eaton *et al.*, 1947; Hamre and Rake, 1947; Kramer and Gordon, 1971; Matsumoto and Manire, 1970; Tamura and

Manire, 1968; Weiss, 1950). Nevertheless, it took over thirty years before Barbour *et al.* identified a mechanism for these effects. Using [^{3}H]-penicillin, three PBPs were detected in both chlamydial RBs and EBs and recent genome sequencing has confirmed their existence in *Chlamydia* (Barbour *et al.*, 1982; Read *et al.*, 2000; Read *et al.*, 2003; Shirai *et al.*, 2000; Stephens *et al.*, 1998; Thomson *et al.*, 2005). Chlamydial PBP1 and PBP2 are predicted to be class B transpeptidases while the chlamydial PBP3 is likely a carboxypeptidase. The chlamydial PBPs share high sequence homology to *E. coli* PBP2, PBP3 (FtsI) and PBP6 (DacC), respectively, although the PBP2 homologue contains a unique 457 amino acid polypeptide insertion. In *E. coli,* PBP2 is involved in elongation and shape determination of the cell while FtsI is involved in cell division (Goffin and Ghuysen, 1998; Popham and Young, 2003). When *E. coli* is exposed to sublethal concentrations of mecillinam, cell division proceeds although the bacteria is transformed from rod-shaped to cocci as mecillinam has a high specific affinity for *E. coli* PBP2 . Mecillinam also specifically targets PBP1 of *Chlamydia* and exhibits potent antichlamydial activity (Bostock *et al.*, 2004; Hammerschlag and Gleyzer, 1983; Storey and Chopra, 2001). Interestingly, like penicillin, mecillinam treatment leads to the formation of aberrant RBs and the inhibition of cell division suggesting that the chlamydial PBP1 may have a prominent role in both cell shape determination and cell division in *Chlamydia*.

Based on the absence of a class A PBP and monofunctional glycosylases in the *Chlamydia* genomes, Ghuysen and Goffin have postulated that *Chlamydia* synthesizes a glycanless cell wall consisting of peptide repeats whose synthesis is sensitive to penicillin (Ghuysen and Goffin, 1999). In *Bacillus subtilis* however, class A PBPs were believed to be the only enzymes that exhibited glycosylase activity yet surprisingly, a *B. subtilis*

mutant lacking all class A PBPs and Mgts is viable and produces a relatively normal PG structure suggesting that an unidentified enzyme(s) also has glycosylase activity (McPherson and Popham, 2003; Popham and Young, 2003). Similar enzymes may exist in *Chlamydia.* The enzymatic characterization of chlamydial PBPs remains an interesting and open avenue of research especially in understanding the role of the 457 amino acid insertion in the PBP2 homologue and the role that the PBP2 and FtsI homologues play in chlamydial cell division in the absence of FtsZ (discussed below).

Regulation of PG synthesis in Chlamydia.

Because PG synthesis is essential to the growth of bacteria, regulation of cell-wall synthesis is crucial especially under conditions of environmental changes and nutritional stress. Despite the need for remodeling the PG structure as the growth environment changes, little is known regarding the control and regulation of PG synthesis in bacteria. During transition from logarithmic to stationary growth of *E. coli,* autolysis and cross-linkage of the PG structure is altered. While the expression of some of the PG synthesis enzymes is regulated by RpoS, a sigma factor essential for survival during prolonged periods of stationary growth, autolysis and cross-linkage of the PG structure is RpoS-independent suggesting that other factors are involved in growth phase restructuring of the PG (Dougherty and Pucci, 1994). The transition of the facultative intracellular organism *Salmonella* from growth in the free-living environment to growth in a eukaryotic cell leads to significant restructuring of the cell wall (Quintela *et al.*, 1997). Compared to PG from free-living *Salmonella,* the cell wall of the intracellular *Salmonella* consists of short, cross-linked glycan chains suggesting a greater role for *N*-acetylmuramyl-L-alanine amidase.

Furthermore, the cell wall has a greater number of peptide cross-linkages overall with numerous *meso*-DAP to *meso*-DAP linkages. However, the mechanisms by which these changes in PG synthesis occur are undefined.

The availability of gene arrays for different bacterial species has begun to enhance our knowledge of how an organism survives under different growth conditions such as environmental or nutritional stress. These arrays allow whole genome transcription profiles to be compared between bacteria grown under different conditions. Using this technology, *murF*, a gene in the *dcw* cluster encoding the D-Ala-D-Ala adding PG synthesis enzyme, and *dadX*, encoding the catabolic racemase, were found to be up-regulated in *E. coli* under conditions of oxidative stress (Pomposiello *et al.*, 2001). With the availability of > 200 fully sequenced bacterial genomes, the ability to scan complete genomes for binding sites of regulatory elements has also enhanced our understanding of bacterial adaptation to specific environments. In *Mycobacterium smegmatis*, IdeR is an iron-responsive DNA binding regulator involved in iron acquisition. Scanning the *M. tuberculosis* genome for IdeR regulator DNA binding sites identified a putative IdeR binding site for *murB* and a penicillin binding protein gene suggesting that PG synthesis may be regulated at the level of transcription under conditions when *Mycobacteria* encounter low iron concentrations (Gold *et al.*, 2001).

In *Chlamydia*, regulation of PG synthesis can be envisioned to be crucial during transition from RBs to EBs as well as under conditions that induce a state of persistence or stationary growth. Under conditions of iron-limitation, *C. trachomatis* development and infectivity is impaired and two dimensional polyacrylamide gel electrophoresis identified one iron-induced and at least 19 iron-repressed proteins (Raulston, 1997). In many

bacteria, protein expression under conditions of iron limitation is regulated at the level of transcription by the ferric uptake regulator (Fur) (Hantke, 2001). Wyllie and Raulston identified and characterized an ORF in *C. trachomatis* (CT296) that encodes a distant relative of Fur which they termed a chlamydial divalent cation-dependent regulator A (DcrA) (Wyllie and Raulston, 2001). Recently, chlamydial genomic sequences recognized by both *E. coli* Fur and the chlamydial DcrA were identified. Interestingly, a Fur/DcrA recognition sequence was found upstream of *murD* (Rau *et al.*, 2005). It is unclear whether DcrA would positively or negatively regulate *murD* transcription although this discovery suggests that regulation of PG synthesis occurs in *Chlamydia* under adverse growth conditions.

Roles for PG in *Chlamydia*.

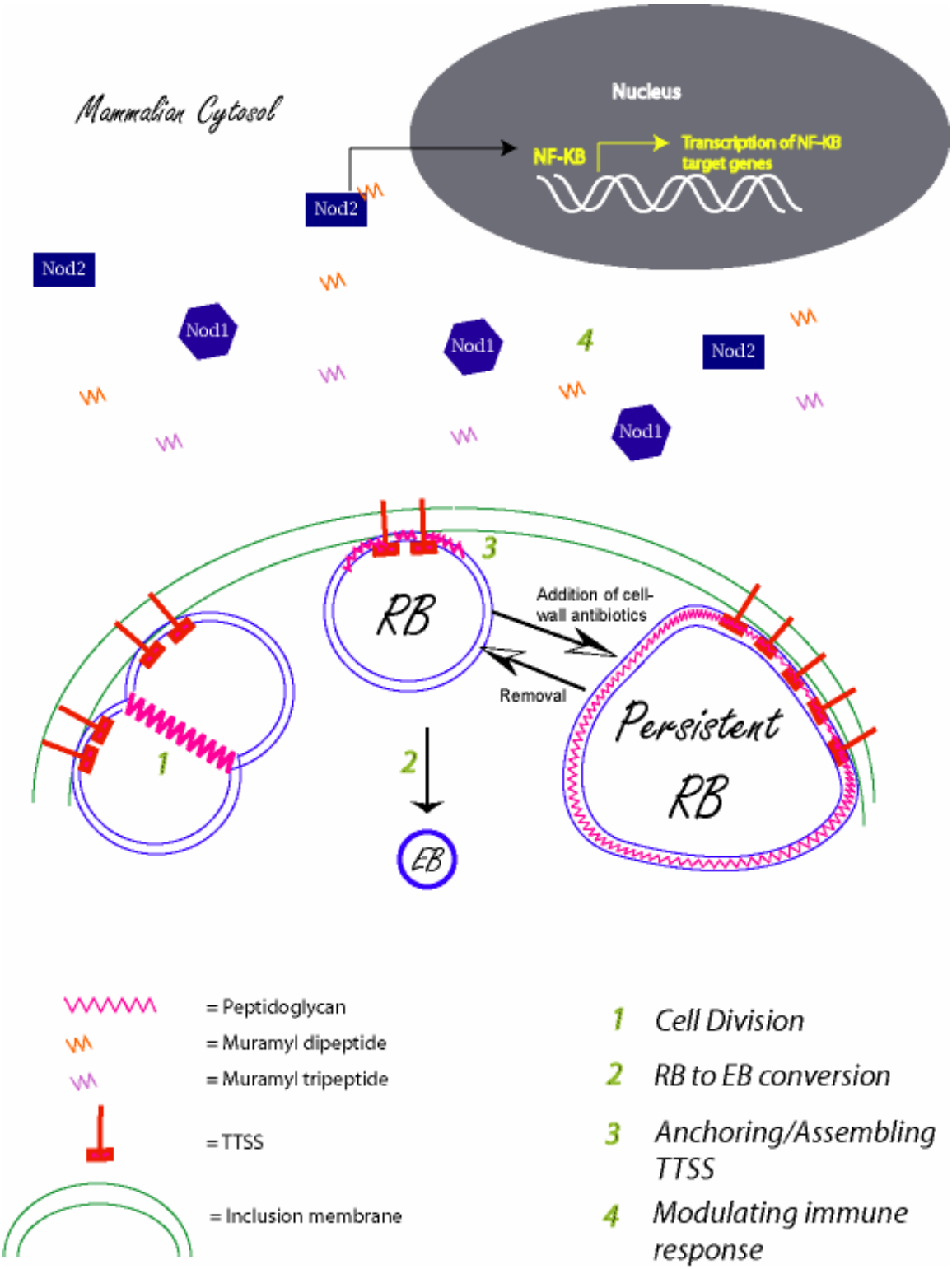
The main reason that PG has yet to be detected in *Chlamydia* most likely reflects the fact that all but one attempt has tried to identify PG in EBs (Chopra *et al.*, 1998). As discussed previously, cross-linking of cysteine-rich proteins in the EB cell envelope likely serves as a functional equivalent for PG in EBs but what about in RBs where the disulfide bonds of the cell envelope proteins are reduced? When *Chlamydia*-infected cells are treated with antibiotics that target cell-wall synthesis, chlamydial cell division is arrested and the RBs become large and aberrant suggesting that PG plays a role in RB development and thus should be detected in RBs. Moreover, this morphology is in contrast to other bacteria that lyse when steps involved in the assembly of the PG subunit are inhibited unless the antibiotic treatment occurs in an isotonic medium. Therefore, it is likely that the inclusion provides a relatively isotonic niche for *Chlamydia* and PG probably plays a

minimal role in maintaining the osmotic integrity of the bacteria. What role then would PG play in chlamydial cell biology?

Cell Division

Another paradox of *Chlamydia* biology is the ability of the bacteria to divide by binary fission in the absence of an FtsZ homologue. Binary fission in bacteria such as *E. coli* proceeds by the formation of a cytokinetic ring at the midcell (Lowe *et al.*, 2004). This ring structure (Z-ring) is formed by the polymerization of the tubulin-like FtsZ protein, which is highly conserved within the bacterial kingdom. After formation of the Z-ring other proteins (ZipA, FtsA, FtsK, FtsQ, FtsW, FtsI, and FtsN) are recruited to the midcell. Of these proteins FtsI (PBP3) and FtsW are essential for PG synthesis. Although many of the recruited Z-ring proteins are not as highly conserved among bacteria as FtsZ, all bacteria that contain PG have an FtsI and FtsW homologue, including *Chlamydia*. In the absence of FtsZ the formation of a PG-ring at the midcell of dividing RBs may represent a functional equivalent of the Z-ring (Figure D.1). Underlying this notion is the observation that antibodies generated against Ribi trivalent adjuvant recognize an antigen (SEP) present at the midcell of dividing RBs (Brown and Rockey, 2000). The SEP antigen is most likely similar to the *Mycobacteria* cell-wall component of the adjuvant. SEP in RBs displays a distribution pattern similar to Z-ring formation in other bacteria. As the RBs transition to EBs, the distribution of SEP becomes diffuse and irregular suggesting that an unknown trigger event to arrest cell division and initiate the RB to EB conversion may globally regulate enzymes involved in PG remodeling and degradation. Interestingly, in the presence of either ampicillin or DCS, which arrest cell division and lead to aberrant RBs,

Figure D.1. Proposed roles of PG in *Chlamydia* biology. EB = elementary body, **RB** = reticulate body; **TTSS** = Type III secretion system. Persistent RBs have only been observed under *in vitro* growth conditions and may not be a reflection of *in vivo* growth under similar conditions.


Mammalian Cytosol
Nucleus
NF-KB
Transcription of NF-KB target genes
Nod2
Nod2
Nod1
Nod1
Nod1
Nod2
4
3
RB
Addition of cell-wall antibiotics
Removal
Persistent
RB
1
2
EB
= Peptidoglycan
= Muramyl dipeptide
= Muramyl tripeptide
= TTSS
= Inclusion membrane
1 Cell Division
2 RB to EB conversion
3 Anchoring/Assembling TTSS
4 Modulating immune response

SEP no longer forms ring structures at the midcell (Brown and Rockey, 2000). Rather, SEP appears to redistribute around the periphery of the cell.

How PG synthesis is directed to the midcell of dividing RBs remains an enigma based on what is known about cell division in other bacteria. However, one possibility is that MreB, an actin-like homologue, may target PG synthesis to the midcell of the RB. In *E. coli*, *B. subtilis*, and *Caulobacter crescentus*, MreB forms helical-like structures that transverse along the longitudinal axis of the bacteria (Figge *et al.*, 2004; Jones *et al.*, 2001; Shih *et al.*, 2003). In *Caulobacter*, the distribution of MreB changes during the cell cycle from spiral-like structures over the length of the cell early in the cell cycle to being concentrated at the mid-cell region during division and back to a spiral-like distribution at the completion of cell division (Figge *et al.*, 2004). Furthermore, localization of PBP2, the PBP responsible for cell shape, is dependent on MreB for localization, however, PBP2 does not localize to the mid-cell at the time of cell division. Together, these data that MreB may play a role in coordinating the switch from longitudinal to septal PG assembly.

RB→EB conversion

Completion of the developmental cycle of *Chlamydia* requires that RBs convert back to EBs in order to infect an adjacent cell and initiate a new round of the developmental cycle. Beside blocking cell division, PG-synthesis inhibitors also hinder the conversion of RBs to EBs suggesting that PG plays a role in this conversion (Chopra *et al.*, 1998; Matsumoto and Manire, 1970; Moulder *et al.*, 1963; Moulder, 1993). As noted above, in the presence of cell-wall inhibitors, SEP is redistributed from the midcell to the periphery in aberrant RBs and in *C. trachomatis*, the periphery SEP accumulates in very

high concentrations (Brown and Rockey, 2000). Under these conditions, the PG structure is likely modified as transpeptidation is inhibited directly and indirectly by β-lactams and DCS, respectively. Ultimately, this modified structure may not be recognized by PG hydrolases to be degraded. The presence of the altered PG structure may thereby directly block RB to EB conversion by sterically hindering the cross-linking of cysteine-rich proteins in the outer membrane (Figure D.1). It is also possible that the inhibition of RB to EB conversion by cell-wall-targeting antibiotics may be an indirect effect of arresting cell division. *In vitro* environmental stress and nutrient-limitation, including IFN-γ exposure and amino acid and iron deficiency, are also known to arrest the growth of *Chlamydia* and produce the ultrastructural traits described with antibiotic treatment (Hogan *et al*., 2004). Although *Chlamydia* is viable under these conditions, they exhibit a loss of infectivity and chlamydial inclusions are often small containing few bacteria. In most cases, once the stress is removed, *Chlamydia* begins to divide and proceed through the developmental cycle. Possibly, the conversion of RB to EB during the chlamydial developmental cycle is related to the number of bacteria or the compactness of the bacteria in the inclusion. Under all the stress conditions including antibiotic treatment, the inhibition of cell division leads to fewer numbers of bacteria within the inclusion which also leads to less compact RBs.

Assembly and Anchoring of the Putative Type III Secretion (T3S) Apparatus

Hsai *et al.* first discovered the presence of genes encoding for a T3S system in *Chlamydia* (Hsia *et al.*, 1997). This discovery was later confirmed when the first genome of *Chlamydia* was sequenced. T3S systems are highly organized needle-like structures found in numerous gram-negative pathogens (Ghosh, 2004). T3S systems are involved in

contact-dependent delivery of bacterial effector proteins to eukaryotic cells and are often essential to the virulence of the pathogen. Proteins of the chlamydial T3S apparatus have been detected in both EBs and RBs and numerous groups have identified chlamydial proteins in the inclusion and host-cell cytosol that are secreted through the heterologous T3S system of *Yersinia*, *Shigella* and *Salmonella* indirectly supporting the functionality of the chlamydial T3S system (Clifton *et al.*, 2004; Fields and Hackstadt, 2000; Fields *et al.*, 2003; Ho and Starnbach, 2005; Lugert *et al.*, 2004; Shaw *et al.*, 2002; Skipp *et al.*, 2005; Subtil *et al.*, 2001; Vandahl *et al.*, 2001). Recently, Pucciarelli and García-del Portillo provided evidence for the association of PG and proteins of one of the *Salmonella* T3S systems, specifically InvH, PrgH and PrgK (Pucciarelli and Garcia-del Portillo, 2003). Alterations within the PG layer eliminated these interactions, which subsequently led to reduced rates of protein secretion and invasion into eukaryotic cells by *Salmonella*. They further proposed that the PG interaction with a subpopulation of *Salmonella* InvH modulated the assembly of a functional needle structure. A similar PG-T3S system interaction may occur in *Chlamydia* RBs as a mechanism of modulating assembly of a functional needle or just to anchor the T3S system as it spans from the bacterial cytoplasm to the cytosol of the host cell (Figure D.1). In EBs, anchoring of the T3S apparatus is predicted to occur through interactions with the cross-linked proteins in the cell envelope.

Immune Modulator

In order to detect and ward off microbial pathogens, eukaryotic cells express pattern-recognition receptors (PRR) that recognize pathogen-associated molecular patterns (PAMP). PAMPs are often conserved structural components of the microbe and include

the bacterial structures lipopolysaccharide (LPS), lipoteichoic acid, flagellin and PG. The best studied class of PRRs is the Toll-like receptor (TLR) family. Upon recognition of their ligands, the transmembrane TLRs interact with cytosolic factors to mediate immune responses via the activation of several protein kinases and transcriptional factors such as NF-κB and ERK (Kawai and Shizuo, 2005). NF-κB-activated signaling via TLRs is dependent upon the adaptor protein, MyD88 and leads to the secretion of proinflammatory cytokines and chemokines. These molecules are crucial to innate immune responses and adaptive immune responses. The best characterized TLRs are TLR4, which recognizes the lipid A portion of LPS and TLR2, which recognizes lipoproteins. It has long been presumed that PG was also a ligand for TLR2, however recent evidence suggests that TLR2 is not activated by PG but rather by lipoproteins that are present in partially purified PG preparations (Travassos *et al.*, 2004).

The nucleotide-binding oligomerization domain (Nod) family of PRRs has received much attention in recent years due to its implication in chronic inflammatory diseases (Carneiro *et al.*, 2004; Inohara *et al.*, 2005). These cytosolic proteins are thought to play an essential role in sensing intracellular bacteria through the recognition of distinct motifs within PG. Nod1 is ubiquitously expressed in eukaryotic cells and detects the naturally-occurring muropeptide, GlcNAc-MurNAc-tripeptide. Specifically, Nod1 signaling requires the presence of an unmodified *meso*-DAP at the terminal end of the muropeptide. Thus, Nod1 is thought to detect primarily intracellular gram-negative bacteria given that *meso*-DAP present in a few gram-positive organisms is modified and PG fragments from these organisms are weak stimulators of Nod1. In contrast, Nod2 is primarily found in antigen presenting cells and recognizes naturally occurring GlcNAc-MurNAc-dipeptide. Because

this muropeptide is structurally conserved in both gram-negative and gram-positive organisms, Nod2 is believed to be a general sensor of intracellular pathogens. In response to sensing their respective ligands, Nods, like TLRs, induce the activation of NF-κB, albeit in a MyD88-independent fashion (Inohara *et al.*, 2005). Evidence suggests that Nod signaling also activates the JNK and p38 pathways as well (Girardin *et al.*, 2001) (Kobayashi *et al.*, 2002; Navas *et al.*, 1999). Activation of these pathways leads to the transcriptional activation of proinflammatory cytokines and chemokines (e.g. IL-6, IL-8, TNFα, MIP-1α), hematopoietic growth factors (e.g. G-CSF, GM-CSF) and adhesion molecules.

Chlamydial infections are characterized by chronic (persistent) infections and intense inflammation. This inflammation can be exacerbated upon re-infection, ultimately leading to tissue damage and scarring. The first cells likely to be exposed to *Chlamydia* during a primary infection are the mucosal epithelia of the genital and respiratory tracts and ocular epithelia. Thus, these cells are expected to initiate the early immune response to infection. Rasmussen *et. al*. observed *C. trachomatis*-induced secretion of IL-8, GROα, GM-CSF, and IL-6 from cervical and colonic epithelial cells (Rasmussent *et al.*, 1997). Similarly, *C. pneumoniae* infected human lung epithelial cells secrete IL-8 and IL-6 suggesting a common response to infection in epithelial cells which ultimately leads to NF-κB activation (Entrican *et al.*, 2004). LPS is not the component responsible for the production of these proinflammatory mediators by epithelial cells as these cells are unresponsive to LPS. Unlike what is seen with other invasive pathogens, the cytokine secretion induced by *C. trachomatis* is delayed until 20-24 hours post-infection and requires bacterial protein synthesis suggesting that a chlamydial component synthesized

during RB development, *i. e.* not an EB specific protein, is detected intracellularly (Rasmussent *et al.*, 1997) (Eckmann *et al.*, 1993). *Chlamydia* is also known to activate dendritic cells, smooth muscle cells, endothelial cells, monocytes and macrophages by TLR2- and TLR4-dependent mechanisms. In response to chlamydial infection, macrophages and dendritic cells secrete IFN-γ. IFN-γ plays a protective role during chlamydial infections by controlling the growth of the bacteria. In bone marrow-derived macrophages infected with *Chlamydia*, IFN-γ expression is induced through TLR4-MyD88 dependent and TLR4-MyD88-independent pathways (Rothfuchs *et al.*, 2004). Both pathways are critical and cooperative and lead to the activation of NF-κB. Expression of TNF-α, IL-1 and IL-6 mRNA does not require TLR4 or MyD88 in these macrophages suggesting that these two pathways are not completely analogous.

It is likely that *Chlamydia* utilizes PG to modulate the immune system via the Nod signaling pathway to provide a balance between inflammation and persistence (Figure D.1). In support of this notion, Opitz *et. al.* recently demonstrated that Nod1 and Nod2 recognized intracellular *C. pneumoniae* and mediated NF-κB activation in a MyD88-independent fashion in infected human endothelial cells (Opitz *et al.*, 2005). Furthermore, they demonstrated that Nod1 was essential for *Chlamydia*-induced IL-8 production by these cells. IL-8 secretion by endothelial cells was detected at 24 hours post-infection suggesting that like in epithelial cells, a chlamydial factor must be released into the cytosol. Although *Chlamydia* stimulates cytokine responses in both macrophages and monocytes via TLR4, chlamydial LPS does not signal as effectively as LPS from other gram-negative bacteria. This poor stimulatory effect from chlamydial LPS is related to the difference in the acylation of chlamydial LPS compared to other LPS moieties. LPS and MurNAc-

dipeptide synergistically cooperate in the expression of cytokines. In fact, LPS upregulates the expression of Nod2 and other molecules in this signaling pathway while MurNAc-dipeptide upregulates MyD88 expression (Inohara *et al.*, 2005; Marriott *et al.*, 2005). During its evolution, *Chlamydia* may have altered its LPS structure such as to limit the upregulation of components of the Nod signaling pathway thereby modulating the expression of IFN-γ to a level that reduces but does not eradicate the growth of the organism thereby tipping the balance of disease versus persistence toward persistence. In *Salmonella*, a new paradigm is emerging regarding persistence. It has been postulated that the bacteria may intentionally limit proliferation within host cells by stimulating the host defenses which thus modulates the balance between disease and persistence (Tierrez and Garcia-del Portillo, 2005). During chlamydial persistence *Chlamydia* may continue to modulate the immune response by secreting PG into the eukaryotic cell, which can continue to activate the Nod signaling pathways. In support of this concept, Rasmussen *et. al* found little effect on cytokine secretion by *C. trachomatis* infected cells when the cells were treated with penicillin (Rasmussent *et al.*, 1997). As mentioned earlier, penicillin inhibits cell division and RB to EB conversion but not RB metabolism.

The question arises as to how *Chlamydia*, which resides in a membrane-bound vacuole, releases PG into the cytosol. The most likely mechanism for delivery of *Chlamydia* PG fragments into the host cytosol is via a secretion system. *H. pylori* is an extracellular, gram-negative bacteria that inhabits the stomach. This pathogen is associated with chronic gastritis that can lead to severe pathologies such as mucosa-associated lymphoid tissue (MALT) lymphoma and gastric adenocarcinoma. Severe pathology with *H. pylori* strains has been linked to strains that contain a *cag* pathogenicity island, which

encodes for components of a type IV secretion system and is required for IL-8 secretion. Viala *et. al.* demonstrated that IL-8 production by *H. pylori* is dependent on Nod1 and the presence of the *cag* pathogenicity island (Viala *et al.*, 2004). Furthermore, signaling by Nod1 occurs via delivery of PG fragments into epithelial cells by the type IV secretion system. Delivery of PG fragments across the inclusion membrane and into the cytosol may occur in an analogous fashion through the *Chlamydia* T3S system.

Perspective

The greatest contribution in the past decade to the understanding of *Chlamydia* biology has been the chlamydial genome sequencing project. The genetic blueprints of *Chlamydia* have confirmed observations made by numerous researchers but have also raised numerous questions regarding the development of these organisms and the unique niche they occupy. The uncovering of a nearly complete pathway for the synthesis of PG in *Chlamydia* and the subsequent demonstration that many of the enzymes in the pathway are functional will have a significant impact upon the treatment of chlamydial infections. Due to the high economic and social cost of *Chlamydia* infections world-wide, new therapies for the treatment of chlamydial infections are crucial. One pathway that has yet to be fully exploited as a target for intervention is that of PG synthesis of *Chlamydia*. Because PG is unique to bacteria, this pathway offers numerous possibilities for the development of bacteria-specific inhibitors and in the case of the MurC-Ddl fusion, this pathway may lead to *Chlamydia*-specific antibiotics. Many questions still remain regarding how, when, and why PG is synthesized. However, a complete understanding of PG synthesis and the role(s) of PG in *Chlamydia* biology will be achieved when two

additional advances occur: (1) the detection and biochemical characterization of chlamydial PG and (2) the development of a system of genetic manipulation in *Chlamydia.*

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