REVIEW
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Antibiotics, peptidoglycan synthesis and genomics: the chlamydial anomaly revisited

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Background

Peptidoglycan, which forms a sacculus around the majority of eubacteria, is an important cell wall polymer since interference with its synthesis or structure leads to loss of cell shape and integrity (Rogers *et al.*, 1980). Nevertheless, some groups of eubacteria, such as the *Thermatoga* and *Planctomyces*, do not contain peptidoglycan in their walls and in such organisms other polymers impart structural integrity (Stackebrandt *et al.*, 1984; Moulder, 1993; Shah *et al.*, 1997).

Chlamydiae, of which two species, *Chlamydia trachomatis* and *Chlamydia pneumoniae*, are major pathogens for man, are obligate intracellular eubacteria (Schachter, 1992). They represent an interesting group of organisms differentiated from other bacteria by a unique development cycle involving two morphological forms, one adapted to extracellular survival (the infectious elementary body; EB) and the other to intracellular multiplication (the reticulate body; RB) (Moulder, 1991; Schachter, 1992). The development cycle is initiated by attachment and entry of EBs into host cells, which results in the onset of chlamydial metabolic activity. This is followed by transformation of EBs to RBs, RB division and expansion of the chlamydial micro-colony. Finally, RBs differentiate back to infectious EBs which are released following host cell lysis. Within infected monolayers the whole cycle takes approximately 40 h (Moulder, 1991).

In contrast to RBs, which are fragile and pleomorphic, EBs are comparatively rigid and stable (Moulder, 1991; Everett & Hatch, 1995). These differences might be explained by the presence of peptidoglycan in the EB cell wall which would confer shape and mechanical stability. Muramic acid is a unique component of peptidoglycan (Schleifer & Kandler, 1972; Rogers *et al.*, 1980). Although some studies conducted in the 1960s and early 1970s claimed detection of muramic acid in EBs, these

results have not been confirmed in more recent work (Table 1). The earlier methodology, particularly involving colorimetric procedures, is now considered to be unreliable (Fox *et al.*, 1990). It can be concluded that even if EBs do contain peptidoglycan, insufficient quantities of the polymer will be present to form a conventional sacculus imparting structural stability (Fox *et al.*, 1990; Moulder, 1993). Attempts to detect peptidoglycan through the use of anti-peptidoglycan antibodies, or as an electron-dense layer under the EB outer membrane, have also failed to demonstrate its presence (Moulder, 1993; Hatch, 1996). Indeed, the rigidity of EBs is attributed to the presence of two cross-linked, cysteine-rich, proteins (CRPs) in the EB envelope which may functionally substitute for peptidoglycan (Hatch, 1996). Since RBs are relatively fragile and pleomorphic it was not expected that they contain peptidoglycan. There has only been one study with *C. trachomatis* to try to detect peptidoglycan specifically in RBs (Barbour *et al.*, 1982), but no evidence for the polymer in these morphological forms was obtained (Table 1).

Penicillin and other β -lactam antibiotics inhibit the growth of peptidoglycan-containing bacteria by specific inhibition of penicillin-binding proteins (PBPs), which have transpeptidase and carboxypeptidase functions and are involved in the late stages of peptidoglycan synthesis (Rogers *et al.*, 1980; Russell & Chopra, 1996). Eubacteria which lack peptidoglycan are therefore not expected to be sensitive to penicillin. This correlation is observed for the *Planctomyces* and mycoplasmas, both of which are refractory to penicillin and lack peptidoglycan (Roberts, 1992; Moulder, 1993). Surprisingly, however, chlamydiae are an exception to the rule since their development cycle is interrupted by penicillin and other antibiotics known to inhibit peptidoglycan synthesis (How *et al.*, 1984; Moulder, 1993; Gump, 1996). This situation, namely the susceptibility of *Chlamydia* to antibiotics inhibiting peptidoglycan synthesis in the

Table 1. Summary of experimental data arising from the application of muramic acid detection methods in chlamydiae

Organism	Morphological form*	Presence of muramic acid†	Detection method	Reference
<i>Chlamydia psittaci</i>	EB	+	Colorimetric	Jenkin (1960)
	Not defined	+	Colorimetric and conversion to radioactive N-acetylmuramic acid	Perkins & Allison (1963)
	EB	— ^a	During amino acid analysis	Manire & Tamura (1967)
	EB	—	Conversion to radioactive muramicitol	Garrett <i>et al.</i> (1974)
	EB	— ^b	GC-MS	Fox <i>et al.</i> (1990)
<i>Chlamydia trachomatis</i>	Not defined	+	Colorimetric and conversion to radioactive N-acetylmuramic acid	Perkins & Allison (1963)
	Mixture of EB and RB	—	Conversion to radioactive muramicitol	Garrett <i>et al.</i> (1974)
	EB	— ^c	During amino acid analysis	Barbour <i>et al.</i> (1982)
	RB	— ^c	During amino acid analysis	Barbour <i>et al.</i> (1982)
	EB	— ^a	GC-MS	Fox <i>et al.</i> (1990)

* EB, elementary body; RB, reticulate body.

† a, <0.02%; b, ≤0.006%; c, <0.2%.

absence of detectable amounts of the polymer, is known as the chlamydial anomaly (Moulder, 1993). It presents an intriguing biological question which was last addressed in detail in reviews by Moulder (1993) and Hatch (1996). Since the publication of the earlier reviews the complete genome sequence of *C. trachomatis* has become available (Stephens *et al.*, 1998), permitting an extensive re-evaluation of the data. This review revisits the chlamydial anomaly in the context of the new genomic information.

Sensitivity of chlamydiae to inhibitors of peptidoglycan synthesis in the apparent absence of the polymer: the central issue

Penicillin and other β -lactam antibiotics inhibit the growth of chlamydiae (Tamura & Manire, 1968; Kuo & Grayston, 1988; Moulder, 1993; Gump, 1996) and have been used for the therapy of chlamydial infections, particularly when first-line agents are contraindicated (Toomey & Barnes, 1990; Alary *et al.*, 1994). Barbour *et al.* (1982) have suggested that penicillin disrupts chlamydial development by interfering with RB division. This leads to the accumulation of varied structures, including large RB-like forms, the so-called 'penicillin forms', which resume normal development when penicillin is removed (Matsumoto & Manire, 1970; Moulder, 1993). Penicillin does not appear to block the initial conversion of EBs to reticulate forms, although it has been suggested to affect the differentiation of RBs to EBs (Barbour *et al.*, 1982). However, a specific effect on differentiation has not been proven.

The effect of penicillin on chlamydial development has been related to the presence, at least in *C. trachomatis*, of three PBPs which are presumed to be the targets of

penicillin action in chlamydial species (Barbour *et al.*, 1982; Moulder, 1993). PBPs are present in both RBs and EBs (Barbour *et al.*, 1982), but their accessibility to penicillin appears to be lower in EBs than RBs (Barbour *et al.*, 1982), which may account for the failure of penicillin to inhibit the initial EB transformation.

By analogy with bacteria that contain peptidoglycan, it could be assumed that chlamydial PBPs are involved in transpeptidase and carboxypeptidase reactions associated with a peptidoglycan sacculus and involving recognition of peptides containing D-alanyl-D-alanine (Massova & Mobashery, 1998). However, as discussed in the previous section, numerous attempts to detect peptidoglycan in chlamydiae have consistently failed to demonstrate its occurrence. The genus *Chlamydia* therefore may occupy a unique evolutionary position amongst bacteria in possessing PBPs, but no peptidoglycan.

Moulder (1993), among several explanations, has suggested that penicillin inhibits the transpeptidase or carboxypeptidase activities of the chlamydial PBPs, but that the D-alanyl-D-alanine peptide side chains involved are attached to a molecule other than muramic acid. Nevertheless, chlamydiae are also sensitive to other antibiotics known to interfere with peptidoglycan biosynthesis (How *et al.*, 1984; Moulder, 1993). These include D-cycloserine, which inhibits both alanine racemase (*alr* product) and D-alanyl-D-alanine ligase (*ddl* product), and bacitracin, which complexes with the membrane-bound pyrophosphate form of the undecaprenyl (C₅₅-isoprenyl) lipid carrier molecule that remains after the N-acetylglucosamine-N-acetylmuramic acid (disaccharide)-peptide unit has been transferred to the nascent peptidoglycan chain (Rogers *et al.*, 1980). The activity of D-cycloserine against

chlamydiae does indeed point to the biosynthesis of peptidoglycan in these organisms and susceptibility to bacitracin is at least consistent with the presence of the polymer, although this antibiotic also inhibits lipopolysaccharide synthesis (Rogers *et al.*, 1980). The observations described above highlight the chlamydial anomaly, namely the sensitivity of *Chlamydia* to inhibitors of peptidoglycan synthesis in the absence of sufficient quantities of the polymer to form a conventional sacculus around the cell.

The chlamydial genome

Recently, sequencing of the *C. trachomatis* genome has been undertaken at the University of California at Berkeley and Stanford University, USA (Stephens *et al.*, 1998). Do the genomic data cast any light on the chlamydial anomaly? Can the structural genes for the chlamydial PBPs be identified and if so what is their relationship to other PBPs? Is there evidence for homologues of *alr*, *ddl* and other genes involved in peptidoglycan biosynthesis or metabolism? As discussed in the following sections, the recent genomic data provide compelling evidence that *Chlamydia* spp. have the capacity to synthesize peptidoglycan. Since inhibitors of peptidoglycan synthesis interfere with RB division and the transition from RBs to EBs, it is concluded that the polymer participates in these processes. The proposed role of peptidoglycan in chlamydial division implies a novel function for the

polymer which may be related to the apparent absence of *ftsZ* in *Chlamydia*.

PBPs and their molecular features

The genome database identifies three ORFs (D682, D270 and D551) (Table 2) whose products have homology to known PBPs (Massova & Mobashery, 1998). Since three PBPs were originally identified in *C. trachomatis* by direct labelling of organisms with radioactive penicillin (Barbour *et al.*, 1982), it is reasonable to conclude that these correspond, respectively, to ORFs D682, D270 and D551. Nevertheless, there are large discrepancies in the molecular masses of the proteins predicted from the DNA sequences (124 000 Da; 73 430 Da; 49 320 Da) (Table 2) and their apparent molecular masses (88 000 Da; 61 000 Da; 36 000 Da) when detected as PBPs (Barbour *et al.*, 1982).

Analysis of the amino acid sequence of PBP1 (ORF D682) indicates that it can be classified as a high-molecular-mass class B PBP in the scheme recently published by Massova & Mobashery (1998). These proteins are typically bifunctional enzymes with two domains, an N-terminal transglycosylase and a C-terminal transpeptidase, preceded by a hydrophobic N-terminus. The penicillin-sensitive transpeptidase activity leads to the formation of cross-links within the peptidoglycan whereas the penicillin-insensitive transglycosylase activity is responsible for polymerization of disaccharide-peptide units (Rogers *et al.*, 1980).

Table 2. Homologues of proteins involved in, or associated with, peptidoglycan synthesis detected in *C. trachomatis* serotype D (Stephens *et al.*, 1998)

Protein	Function in <i>E. coli</i>	Molecular mass (Da) of subunit	ORF
PBP1	Transglycosylase-transpeptidase	124 000	D682
PBP2	Transglycosylase-transpeptidase	73 400	D270
PBP3	Carboxypeptidase	49 300	D551
MurA	UDP-N-acetylglucosamine enolpyruvyltransferase	48 400	D455
MurB	UDP-N-acetylglucosaminyl-3-enolpyruvate reductase	31 700	D831
MurC and DdlA	L-Alanine-adding enzyme and D-alanine-D-alanine ligase	89 230	D762*
MurD	UDP-N-acetylmuramoyl-L-alanine:D-glutamate ligase	46 100	D758
MurE	meso-Diaminopimelate-adding enzyme	53 300	D269
MurF	D-Alanyl-D-alanine-adding enzyme	49 300	D756
MurG	UDP-N-acetylglucosamine-N-acetylmuramyl (pentapeptide) pyrophosphoryl-undecaprenol-N-acetylglucosamine transferase	38 400	D761
MraY	UDP-N-acetylmuramoyl (pentapeptide)-undecaprenyl-phosphate phosphatase	37 100	D757
GlmU	N-Acetylglucosamine-1-phosphate uridylyltransferase	22 300	D629
MreB	Rod-shape-determining protein	39 500	D709
RodA	Required for PBP expression	42 300	D726
FtsW	Required for PBP expression	42 100	D760
AmiA	N-Acetylmuramoyl-L-alanine amidase	28 700	D268
AmiB	N-Acetylmuramoyl-L-alanine amidase	22 500	D601
AmiD	N-Acetylmuramoyl-L-alanine amidase	53 600	D003
Ami	N-Acetylmuramoyl-L-alanine amidase	27 200	D759

* *murC* and *ddlA* are recorded in the database as a single ORF.

Chlamydial PBP2 (ORF D270) is also a member of the high-molecular-mass class B enzymes (i.e. a bifunctional transglycosylase–transpeptidase), whereas PBP3 (ORF D551) is predicted to be a low-molecular-mass class A PBP showing carboxypeptidase activity. Since these proteins bind penicillin in RBs (Barbour *et al.*, 1982), i.e. the metabolically active forms susceptible to penicillin *in vivo* (Barbour *et al.*, 1982; Moulder, 1993), it is reasonable to assume that they are engaged in peptidoglycan synthesis, which in turn must be essential for RB development.

Other genes known to have a role in peptidoglycan synthesis and metabolism or encoding products associated with the polymer

In addition to identification of PBPs, homologues of many genes known to have a role in the biosynthesis of bacterial peptidoglycan have been identified in the *C. trachomatis* genome database (Table 2). These range from genes encoding enzymes involved in the synthesis of the muramyl-peptide unit (*glmU*, *ddlA*, *murA–murF*) and its attachment (*mraY*) to the undecaprenyl lipid carrier, to the transferase (*murG*) which catalyses the addition of UDP-*N*-acetylglucosamine to form undecaprenyl–disaccharide–peptide (van Heijenoort, 1996). Another similarity to organisms known to synthesize peptidoglycan is exhibited by the clustering and order of chlamydial *murF*, *mraY*, *murD*, *ftsW*, *murG* and *murC/ddl* (Fig. 1), which is analogous to the presence and relative positions of these genes in the 2 min region of the *Escherichia coli* chromosome (van Heijenoort, 1996). However, correlation with the *E. coli* 2 min region is not absolute since *murE* in *E. coli* maps to this region, but does not appear to be located near the *murF–murC/ddl* genes in *C. trachomatis*, and *murD* and *ftsW* in *C. trachomatis* are separated by *ami* (Fig. 1).

The genes for peptidoglycan biosynthesis identified in *C. trachomatis* (Table 2) imply that these organisms produce a peptidoglycan that may be related to that synthesized in *E. coli* (Schleifer & Kandler, 1972; Rogers *et al.*, 1980). Further indication for the presence of peptidoglycan in *C. trachomatis* is suggested by the finding that the organism contains a gene (ORF D600)

homologous to *pal* (*excC*) in *E. coli* which encodes a peptidoglycan-associated lipoprotein. In *E. coli* this lipoprotein is localized in the outer membrane, but firmly (although non-covalently) bound to the peptidoglycan layer of the cell envelope (Chen & Henning, 1987).

Since *C. trachomatis* does not appear to contain alanine racemase (*alr* product), the susceptibility of the organism to D-cycloserine may be mediated at the level of *ddlA*. Absence of *alr* is also consistent with the lack of activity of alaphosphin against *C. trachomatis* (How *et al.*, 1984), but implies that *C. trachomatis* may need to acquire D-alanine. Regarding this point, it is interesting to note that *C. trachomatis* contains two homologues (ORFs D409 and D735) of *dagA* (encoding D-alanine glycine permease; synonym *cycA*), the product of which is a transport protein responsible for accumulation of D-alanine (and cycloserine) in *E. coli* and other bacteria (Robbins & Oxender, 1973; MacLeod & MacLeod, 1986, 1992). The presence of two similar genes encoding membrane proteins with D-alanine transport capacity in *C. trachomatis* may reflect dependency upon an exogenous source of D-alanine for peptidoglycan synthesis in the absence of *alr* which would be able to convert L-alanine to D-alanine. The presence of *dagA* homologues in *C. trachomatis* would be consistent with acquisition of D-alanine from mammalian cells. Although D-amino acids, including D-alanine, have not formally been identified in mammals, their presence in mammalian cells appears likely (Kreil, 1997).

In addition to genes capable of synthesizing peptidoglycan, *C. trachomatis* also appears to contain several amidases (encoded by ORFs D759, D268, D601 and D003; Table 2) with homology to enzymes that hydrolyse the link between *N*-acetylmuramyl residues and L-amino acids in bacterial peptidoglycan (Holtje & Tuomanen, 1991; van Heijenoort, 1996). Such enzymes are thought to permit expansion of the cell wall during bacterial growth by controlled cleavage of peptidoglycan (Holtje & Tuomanen, 1991; van Heijenoort, 1996). Furthermore, *C. trachomatis* possesses homologues of three other genes, *mreB*, *rodA* and *ftsW* (Table 2), which are associated with peptidoglycan biosynthetic activities in other bacteria (Wachi & Matsuhashi, 1989; Lutkenhaus & Mukherjee, 1996).

Fig. 1. Comparison of the genomic regions surrounding the *murF* gene in *E. coli* and *C. trachomatis*. Preliminary data indicate that *murC* and *ddl* in *C. trachomatis* derive from a single gene (see text). The boxes marked 'ORF' indicate ORFs encoding products of unknown function. The unfilled box indicates a non-coding region. Not drawn to scale.

Are the chlamydial genes encoding peptidoglycan biosynthetic and metabolic activities cryptic?

Genes present in an organism but not expressed are defined as cryptic (Bender, 1996) and several examples of such genes have been identified or predicted in bacteria (Bender, 1996; Jensen, 1996; Pollack, 1997; Baltz *et al.*, 1998). Could the apparent absence of peptidoglycan in *Chlamydia* be explained on the basis that one or more of the genes potentially encoding products with peptidoglycan biosynthetic activity are cryptic?

Evidence for the occurrence of early and late stages of peptidoglycan synthesis arises from the susceptibility of *Chlamydia* spp. to cycloserine and penicillin, which is consistent with the expression of DdIA and PBPs, involved, respectively, in the early and late biosynthetic steps. Despite this evidence for the occurrence of early and late stages of peptidoglycan synthesis, is it possible that a key enzyme involved at an intermediate stage of the biosynthetic pathway is cryptic, resulting in failure to synthesize a complete peptidoglycan molecule? This appears to be ruled out on the basis of the activity of PBPs which require as substrates disaccharide-peptides that have been inserted into nascent peptidoglycan by transglycosylation (Rogers *et al.*, 1980). Finally, the genes involved in peptidoglycan synthesis and metabolism in *C. trachomatis* (Table 2) occupy 1.8% of the genome. In view of the high proportion of the genome occupied by these genes, their maintenance would seem unlikely if they were cryptic.

What is the role of peptidoglycan in chlamydiae and is it degraded before the onset of EB formation?

The recent genomic data provide compelling evidence that *Chlamydia* spp. have the capacity to synthesize peptidoglycan. However, if only analytically undetectable amounts of peptidoglycan are present, the polymer cannot have a role in maintaining the structural integrity of EBs or RBs. In the case of the halophilic bacterium *Rhodospirillum salexigens*, which also has insufficient peptidoglycan to form a structural sacculus, it has been suggested that the polymer occurs in patches around flagellar bases (Evers *et al.*, 1986), which might imply a role in the stabilization or anchorage of surface appendages to the cell envelope. However, such a role in *Chlamydia* would appear unlikely in view of the effects of penicillin and cycloserine on cell division.

We are thus left with the intriguing possibility that in *Chlamydia* the primary role of peptidoglycan is in RB division and possibly the transition from RBs to EBs prior to their release from the infected cell. Analysis of ORFs in the *C. trachomatis* genome database has failed to reveal a homologue of *ftsZ* (Stephens *et al.*, 1998), which is highly conserved in other bacteria where it has an essential role in cell division (Lutkenhaus & Mukherjee, 1996). The absence of *ftsZ* in *Chlamydia*

appears to be unique amongst bacteria, which may in turn require an atypical role for peptidoglycan in cell division.

The observation that RBs apparently lack peptidoglycan is based on the results of a single attempt to detect the polymer in this morphological form (Table 1). We predict that peptidoglycan is indeed present in RBs and that further experiments are justified to confirm its presence. However, since several attempts have consistently failed to detect peptidoglycan in EBs (Table 1), we believe that a consensus is emerging that the polymer is absent from these chlamydial forms. Consequently, in the transition from RBs to EBs, the amidases, whose genes have been identified in the chlamydial genome (Table 2), may be activated to degrade peptidoglycan, thereby explaining the absence of detectable quantities of the polymer in EBs.

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