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 Thermatogae 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

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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 & Manrie, 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 (air 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

<table>
<thead>
<tr>
<th>Organism</th>
<th>Morphological form*</th>
<th>Presence of muramic acid†</th>
<th>Detection method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlamydia psittaci</td>
<td>EB</td>
<td>+</td>
<td>Colorimetric</td>
<td>Jenkin (1960)</td>
</tr>
<tr>
<td></td>
<td>Not defined</td>
<td>+</td>
<td>Colorimetric and conversion to radioactive N-acetylmuramic acid</td>
<td>Perkins &amp; Allison (1963)</td>
</tr>
<tr>
<td></td>
<td>EB</td>
<td>−</td>
<td>During amino acid analysis</td>
<td>Manire &amp; Tamura (1967)</td>
</tr>
<tr>
<td></td>
<td>EB</td>
<td>−</td>
<td>Conversion to radioactive muramicitol</td>
<td>Garrett et al. (1974)</td>
</tr>
<tr>
<td></td>
<td>EB</td>
<td>−</td>
<td>GC-MS</td>
<td>Fox et al. (1990)</td>
</tr>
<tr>
<td>Chlamydia trachomatis</td>
<td>Not defined</td>
<td>+</td>
<td>Colorimetric and conversion to radioactive N-acetylmuramic acid</td>
<td>Perkins &amp; Allison (1963)</td>
</tr>
<tr>
<td></td>
<td>Mixture of EB and RB</td>
<td>−</td>
<td>Conversion to radioactive muramicitol</td>
<td>Garrett et al. (1974)</td>
</tr>
<tr>
<td></td>
<td>EB</td>
<td>−</td>
<td>During amino acid analysis</td>
<td>Barbour et al. (1982)</td>
</tr>
<tr>
<td></td>
<td>RB</td>
<td>−</td>
<td>During amino acid analysis</td>
<td>Barbour et al. (1982)</td>
</tr>
<tr>
<td></td>
<td>EB</td>
<td>−</td>
<td>GC-MS</td>
<td>Fox et al. (1990)</td>
</tr>
</tbody>
</table>

*EB, elementary body; RB, reticulate body.
†a, <0.02%; b, ≤0.006%; c, <0.2%.
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 lipopoly saccharide 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 saccus 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.

PBP 

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 (124000 Da; 73430 Da; 49320 Da) (Table 2) and their apparent molecular masses (88000 Da; 61000 Da; 36000 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)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function in E. coli</th>
<th>Molecular mass (Da)</th>
<th>ORF of subunit</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBP1</td>
<td>Transglycosylase–transpeptidase</td>
<td>124000</td>
<td>D682</td>
</tr>
<tr>
<td>PBP2</td>
<td>Transglycosylase–transpeptidase</td>
<td>73400</td>
<td>D270</td>
</tr>
<tr>
<td>PBP3</td>
<td>Carboxypeptidase</td>
<td>49300</td>
<td>D551</td>
</tr>
<tr>
<td>MurA</td>
<td>UDP-N-acetylmuramoyl-N-acetylglucosamine-3-epimerase</td>
<td>48400</td>
<td>D455</td>
</tr>
<tr>
<td>MurB</td>
<td>UDP-N-acetylmuramoyl-N-acetylglucosamine-3-enolpyruvate reductase</td>
<td>31700</td>
<td>D831</td>
</tr>
<tr>
<td>MurC and DdIA</td>
<td>t-Alanine-adding enzyme and d-alanine–d-alanine ligase</td>
<td>89230</td>
<td>D762*</td>
</tr>
<tr>
<td>MurD</td>
<td>UDP-N-acetylmuramoyl-N-acetylglucosamine-3-epimerase</td>
<td>46100</td>
<td>D738</td>
</tr>
<tr>
<td>MurE</td>
<td>meso-Diaminopimelate-adding enzyme</td>
<td>53300</td>
<td>D269</td>
</tr>
<tr>
<td>MurF</td>
<td>N-Alanyl–d-alanine-adding enzyme</td>
<td>49300</td>
<td>D756</td>
</tr>
<tr>
<td>MurG</td>
<td>UDP-N-acetylmuramoyl-N-acetylglucosamine (pentapeptide) pyrophosphoryl-undecaprenol-N-acetylglucosamine transferase</td>
<td>38400</td>
<td>D761</td>
</tr>
<tr>
<td>MrA</td>
<td>UDP-N-acetylmuramoyl (pentapeptide)-undecaprenol-phosphate phosphatase</td>
<td>37100</td>
<td>D757</td>
</tr>
<tr>
<td>GlnU</td>
<td>N-Acetylmuramoyl-1-phosphate uridylyltransferase</td>
<td>22300</td>
<td>D629</td>
</tr>
<tr>
<td>MreB</td>
<td>Rod-shape-determining protein</td>
<td>39500</td>
<td>D709</td>
</tr>
<tr>
<td>RodA</td>
<td>Required for PBP expression</td>
<td>42300</td>
<td>D726</td>
</tr>
<tr>
<td>FtsW</td>
<td>Required for PBP expression</td>
<td>42100</td>
<td>D760</td>
</tr>
<tr>
<td>AmiA</td>
<td>N-Acetylmuramoyl-N-acetylglucosamine amidase</td>
<td>28700</td>
<td>D268</td>
</tr>
<tr>
<td>AmiB</td>
<td>N-Acetylmuramoyl-N-acetylglucosamine amidase</td>
<td>22500</td>
<td>D601</td>
</tr>
<tr>
<td>AmiD</td>
<td>N-Acetylmuramoyl-N-acetylglucosamine amidase</td>
<td>53600</td>
<td>D003</td>
</tr>
<tr>
<td>Ami</td>
<td>N-Acetylmuramoyl-N-acetylglucosamine amidase</td>
<td>27200</td>
<td>D759</td>
</tr>
</tbody>
</table>

* 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-acetylmuramoyl 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 ddIA. 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 & Matsushashi, 1989; Lutkenhaus & Mukherjee, 1996).

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**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 18% 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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References


