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Gaps in the wall: understanding cell wall biology to tackle amoxicillin resistance in *Streptococcus pneumoniae*

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Streptococcus pneumoniae is the most common cause of community-acquired pneumonia, and one of the main pathogens responsible for otitis media infections in children. Amoxicillin (AMX) is a broad-spectrum β-lactam antibiotic, used frequently for the treatment of bacterial respiratory tract infections. Here, we discuss the pneumococcal response to AMX, including the mode of action of AMX, the effects on autolysin regulation, and the evolution of resistance through natural transformation. We discuss current knowledge gaps in the synthesis and translocation of peptidoglycan and teichoic acids, major constituents of the pneumococcal cell wall and critical to AMX activity. Furthermore, an outlook of AMX resistance research is presented, including the development of natural competence inhibitors to block evolution via horizontal gene transfer, and the use of high-throughput essentiality screens for the discovery of novel cotherapeutics.

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Introduction

Amoxicillin (AMX) is the frontline antibiotic for the treatment of bacterial respiratory tract infections such as otitis media and pneumonia. The global leading cause of community-acquired pneumonia and one of the most common causes of otitis media in children is *Streptococcus pneumoniae* (the pneumococcus) [1]. This Gram-positive opportunistic pathogen is an important commensal member of the human nasopharyngeal microflora and while colonization is usually asymptomatic, local spread or invasion of other regions can result in symptomatic infection. In addition, the pneumococcus is capable of crossing epithelial barriers to cause severe invasive pneumococcal disease (IPD), such as sepsis and meningitis, and was responsible for more than 800 000 deaths in 2019 [2]. The pneumococcus has a remarkable genomic plasticity that is driven by a phenomenon called natural competence that allows bacteria to take up and integrate extracellular DNA from its environment, thereby able to rapidly evolve in response to stressful conditions such as antibiotic exposure [3]. As such, antibiotic resistance is evolving rapidly and the high burden of pneumococcal disease, despite vaccine introduction, led to the WHO listing *S. pneumoniae* as a top-12 priority pathogen in 2017. Indeed, it was recently estimated that approximately 600 000 people died in 2019 due to antibiotic resistance-associated pneumococcal infections [4].

AMX is one of the most prescribed antibiotics globally [5]. AMX is a penicillin (PEN)-derived β-lactam antibiotic and highly active toward *S. pneumoniae*, including against isolates with reduced susceptibility toward PEN [6]. Using fluorescently labeled bocillin, it was shown that AMX has a distinct mode of action compared with PEN (see below, [7]). In addition, the high absorption rate of AMX into the bloodstream reduces side effects and increases serum concentrations, making it desirable as an oral treatment. It is commonly prescribed for otitis media and outpatient community-acquired pneumonia with reductions in recommendations for treatment duration, thereby limiting the chance of selection of resistant strains [8]. The current clinical breakpoints for oral AMX treatment recommended by european committee on antimicrobial susceptibility testing (EUCAST) are minimum inhibitory concentration (MIC) < 0.5 μg/mL to be considered fully susceptible and ≥ 1 μg/mL for resistance [9]. Strains with AMX MICs greater than 16 μg/mL were isolated in Romania between 2005 and 2006 [10], and are currently on the rise in Spain, a phenomenon that correlates with increased usage of oral AMX/clavulanic acid (sold as Augmentin) [11]. Clavulanic acid inhibits β-lactamase enzymes, frequently found in Gram-negative pathogens such as *Haemophilus influenzae*, another causative agent of otitis media [1]. These enzymes have not yet been found in *S. pneumoniae*.

AMX usage and consequent resistance evolution occurred in the context of widespread PEN usage and circulation of β-lactam resistance determinants [12]. It is thus impossible to delve into AMX-specific resistance mechanisms and dissemination without first understanding general mechanisms of pneumococcal β-lactam resistance. Here, we examine the fundamental process of cell wall synthesis and its inhibition by β-lactam antibiotics, as well as the resulting downstream effects, including autolysis. We discuss resistance evolution through rampant horizontal gene transfer between *S. pneumoniae* and commensal Streptococcal species. We then summarize the current knowledge of β-lactam resistance determinants in the pneumococcus and discuss how these have changed in response to the shift toward broad clinical usage of AMX.

β-lactam treatment and the cell wall

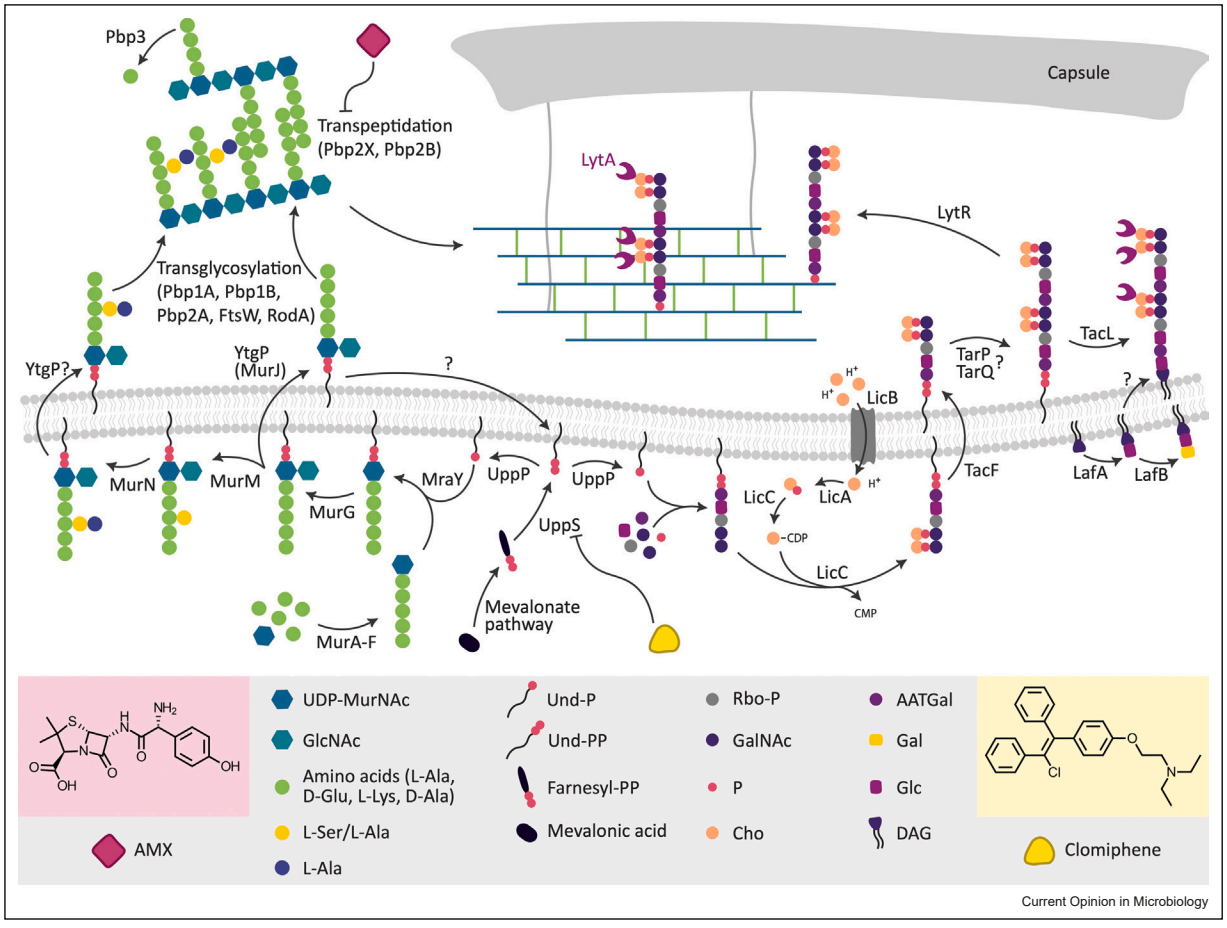
Cell wall synthesis is essential for cell viability, making this pathway an attractive target for antibiotic development [13]. In the pneumococcus, the cell wall is composed of roughly equal quantities of both peptidoglycan (PG) and teichoic acids (TA), both of which play a role in the cellular response to β-lactam treatment (Fig. 1).

PG biosynthesis starts with the mevalonate pathway in which the precursors for the production of all isoprenoids in *S. pneumoniae* are generated [14]. One of those isoprenoids, undecaprenyl phosphate (Und-P), acts as the lipid carrier for the PG precursor lipid II [15]. Lipid-II precursors are synthesized in the cytoplasm, then flipped and exposed on the outside of the membrane, possibly by YtgP, an essential flippase similar to MurJ in Gram-negative bacteria [16,17]. In addition to linear lipid II, branched muropeptides are produced by the aminoacyl-tRNA-dependent ligases MurM and MurN, which add L-Ser-L-Ala or L-Ala-L-Ala to the lysine in the stem peptide [18•]. It is not known whether YtgP can also flip branched muropeptides or whether other flippases are involved (Fig. 1). Incorporation of lipid II into the PG occurs in two steps performed by penicillin-binding proteins (PBPs) and shape, elongation, division, and sporulation (SEDS) proteins FtsW and RodA [19–21]. Transglycosylation, in which precursors are assembled into PG chains, is followed by transpeptidation, where cross-links between neighboring peptides are formed. Of the six PBPs encoded by *S. pneumoniae*, only Pbp2x and Pbp2b are essential under normal growth conditions. These proteins are both high-molecular-weight (HMW) class-B PBPs, with transpeptidase (TP) and PBP dimer domains. In addition, both pneumococcal SEDS proteins FtsW and RodA are essential [22]. Pbp2x is required for septal PG synthesis and depletion results in an elongated phenotype, while Pbp2B is involved in peripheral PG synthesis and depletion leads to short cells [23•]. Pbp1a, Pbp1b, and Pbp2a, HMW class-A PBPs, have both TP and transglycosidase domains and are not essential in normal growth conditions. Pbp3 is a low-molecular-weight D,D-carboxypeptidase that regulates PG synthesis by cleaving the fifth amino acid from pentapeptides already integrated in the PG matrix, reducing available substrate for incorporation of new lipid-II monomers [24].

Septal and longitudinal PG synthesis are maintained in a delicate balance and inhibition of the two essential PBPs is tolerated to differing extents [25,26]. Recent work, using direct Stochastic Optical Reconstruction Microscopy (dSTORM) and 3D-Structured Illumination Microscopy on fluorescent D-amino acid-labeled PG in *S. pneumoniae*, showed spatially ordered PG synthesis with septal PG being synthesized ahead of the peripheral PG, which is inserted into the septal PG after its splitting by septum hydrolases [27••,28]. The serine/threonine kinase StkP, together with adapter proteins DivIVA and GpsB, also plays a crucial role in regulating cell elongation and division [29]. In addition, using sCRilecs-seq (subsets of clustered regularly interspaced short palindromic repeats (CRISPR) interference libraries extracted by fluorescence-activated cell sorting coupled to next-generation sequencing), it was shown that septal PG synthesis is more sensitive to reduced Und-P levels than peripheral PG synthesis, suggesting that the regulation between elongation and division is also controlled at the level of substrate concentration [30].

β-lactam antibiotics mimic the D-ala-D-ala moiety of the muropeptides, which is recognized by the PBPs for transpeptidation, allowing an interaction with the active-site serines of TP and D,D-carboxypeptidase domains [39]. The resulting acylation reaction hydrolyzes the high-energy β-lactam ring to form a covalent penicilloyl-enzyme complex, blocking the active site [40]. Inhibition of transpeptidation is the critical step that stops cell wall synthesis, although almost all β-lactams also exhibit potent inhibition of Pbp3 via the D,D-carboxypeptidase domain [7]. Cell shape is dependent on tight control of transpeptidation and transglycosylation. TP inhibition results in a high concentration of un-crosslinked glycan strands without the structural support of cross-linking, induces nascent PG turnover, and depletes intracellular stocks of cell wall building blocks, ultimately contributing to bacterial death [41]. In addition, it is hypothesized that the uncoupling of transpeptidation and transglycosylation activities combined with the loss of cell wall synthesis quality checking capabilities of other proteins during β-lactam treatment results in the mis-activation of autolytic hydrolases [41]. PG hydrolases are essential for insertion of muropeptides into the existing cell wall structure, septum formation, and daughter cell separation [29]. However, their activity is tightly regulated, and loss of this control leads to explosive lysis (Fig. 2).

Figure 1

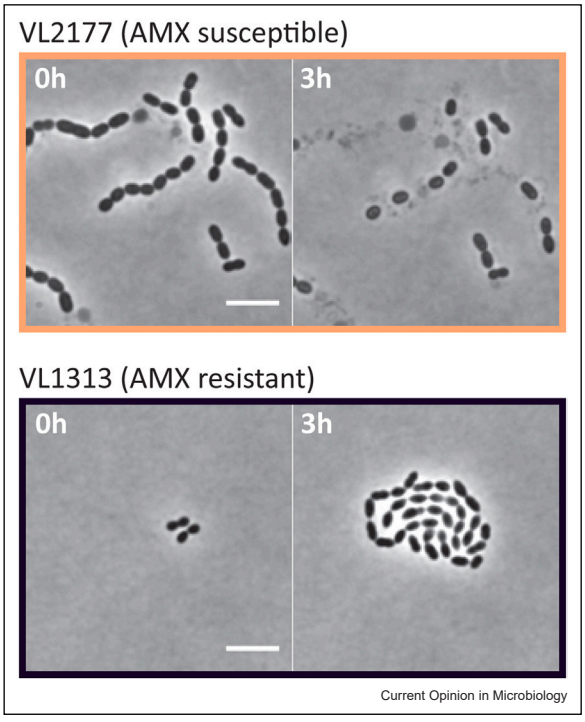


Overview of cell wall synthesis in the pneumococcus. Knowledge gaps in key details regarding pneumococcal cell wall biology are indicated by question marks. Both PG and TA precursors are anchored to the membrane by lipid carrier undecaprenol phosphate (Und-P). Und-P is made from farnesyl-PP precursors produced via the mevalonate pathway [14,15]. Farnesyl-PP is used by UppS to produce undecaprenyl pyrophosphate (Und-PP), which is then dephosphorylated to Und-P by UppP. UppS is inhibited by FDA-approved drug Clomiphene (yellow triangle, structure indicated), a potential cotherapeutic for the resensitization of AMX-resistant pneumococcal infections [30]. Pentapeptides are synthesized from uridine diphosphate-N-acetylmuramic acid (UPD-MurNAc) and amino acids in the cytoplasm by MurA-F. Transfer to Und-P is performed by MraY, then N-acetylglucosamine (GlcNAc) is added by MurG forming Lipid II. Lipid II is either transferred over the membrane by predicted flippase YtgP (MurJ homolog), or additional amino acids are added by MurM and MurN, resulting in branched Lipid II before flipping [18•]. Whether YtgP is the actual pneumococcal Lipid II flippase and can also flip branched Lipid II is still unknown. These precursors are then incorporated into the PG matrix via PBP, RodA, and FtsW-mediated transglycosylation. Und-PP is assumed to be recycled by UppP, but a flippase would also be required for transport back across the membrane. Recent work in Bacillus subtilis and Staphylococcus aureus identified widely conserved proteins UptA and PopT as the missing transporters [31,32], but homology in *S. pneumoniae* remains to be explored. Peptide bridges are formed by transpeptidation, thought to be largely catalyzed by Pbp2b and Pbp2x. Transpeptidase activity is inhibited by AMX (pink square, structure indicated). Pbp3 contributes to PG maturation by cleaving the terminal alanine from Lipid II, blocking further cross-linking. TA precursors are also synthesized in the cytoplasm in a series of membrane-bound steps from N-acetylgalactosamine (GalNAc), 2-acetamido-4-amino-2,4,6-trideoxygalactose (AATgal), glucose (Glc), and ribitol-5-phosphate (Rbo-P) [33]. They are then modified with phosphorylcholine groups by LicC, following proton-coupled choline import by LicB [34], and phosphorylation by LicA [35]. TacF flips phosphorylcholine-modified precursors over the membrane, to be used as substrates for polymerization by TarQ and TarP [22]. As TarP is classified by PFAM as a Wzy-type polymerase with predicted active site outside the cell, we speculate that TarP and TarQ polymerize the TA extracellularly in contrast to previous reports [33]. Multimers are then either attached to a glycolipid anchor by TacL [36], or anchored to the PG matrix presumably by LytR [37], competing for anchor points with capsule tethers [38]. Glycolipid anchors are thought to consist of glucosyl-diacylglycerol (Glc-DAG), synthesized by addition of Glc to diacylglycerol (DAG) via LafA, and exposed on the outside of the membrane by an unknown flippase. Note that the pneumococcus has a second glycolipid GalGlc-DAG produced by LafB (CpoA)-catalyzed addition of galactosyl (Gal) to Glc-DAG. LytA attaches to phosphorylcholine residues on TA at certain stages of growth and is responsible for AMX-induced autolysis via its PG hydrolase activity.

Pneumococcal autolysis

Major pneumococcal autolysin LytA is required for lysis to occur following β-lactam treatment [43]. LytA is an N-acetylmuramoyl L-alanine amidase with a choline-binding domain. LytA dimers bind to choline-decorated TAs, orienting the active site to cleave the amide bond

Figure 2



Cell lysis following AMX treatment. Snapshots from phase-contrast time-lapse imaging of VL2177 (TIGR4, AMX MIC 0.016 μg/mL), a serotype 4, AMX-susceptible laboratory strain, and VL1313 (ST6521, AMX MIC 4 μg/mL), a serotype 11A AMX-resistant clinical isolate, grown in AMX 1 μg/mL [42••]. Dramatic lysis of the AMX-susceptible strain is visible by 3 h of exposure. Micrographs were produced in Gibson et al., 2022.

between glycan strand and peptide in the PG [44] (Fig. 1). TAs are an important structural component of the cell wall. Precursors are assembled in the cytoplasm before being decorated with phosphorylcholine groups by LicC [33]. TacF transports phosphorylcholine-modified TA precursors over the membrane ready for polymerization by TarQ and TarP [22]. It has typically been thought that precursor polymerization occurred in the cytoplasm before flipping, however, based on homology of TarP with Wzy-type polymerases, we would expect the predicted active site to be situated outside the cell. We thus hypothesize that polymerization occurs extracellularly [33]. TA multimers are either anchored onto the PG matrix, presumably by LytR to form wall teichoic acids (WTA) [37] or transferred to a glycolipid anchor by TacL for lipid teichoic acids (LTA) [45]. WTA and LTA pathways thus compete for the same precursors, and the proportions of these two cell wall structures are thought to be important for the regulation of LytA activity [36].

LytA is constitutively expressed through all growth phases, indicating strict regulation at the protein level [46]. LytA is predominantly cytoplasmic during exponential growth and shifts to the extracellular surface in stationary phase [36,47]. How this shift occurs and what factors affect the timing or control of the autolytic activity are not well understood. PEN tolerance is associated with changes to autolysin regulatory elements in clinical isolates, as opposed to complete loss of LytA or its function [48]. This is perhaps not surprising as LytA deletion mutants have attenuated virulence in murine infection models [49]. In some cases, autolysin was still present in lower concentrations than in a wild-type strain, and tolerant isolates were still susceptible to lysis via other induction routes [50]. Although not well studied for AMX, we could expect some cross-tolerance from PEN-tolerant mutants.

Natural competence and amoxicillin resistance evolution in the pneumococcus

The first AMX-resistant strains isolated between 1994 and 1995 had similar MICs for both AMX and PEN [51]. Isolates with higher MICs for AMX than for PEN began to be isolated in France in 1997 [12], while two large cohort studies across Spain between the years of 1998–1999 and 2001–2002 found that 5% of isolates were AMX-resistant, a figure that increased to 20% when only PEN-resistant isolates were considered [52,53]. Pneumococcal strains are characterized by both genetic sequence type and capsule-dependent serotype. Closely related sequence types are clustered into clonal complexes to reflect close relationships. A more comprehensive study of 165 pneumococcal strains isolated from 17 Spanish hospitals between 1998 and 1999 found that 9.8% were AMX nonsusceptible [54,55]. Among the AMX-resistant isolates collected during this time, approximately 78% of them belonged to five clonal complexes notorious for PEN resistance: Spain^{23F}-1, Spain^{6B}-2, Spain^{9V}-3, Poland^{23F}-16, and an England^{14}-9 [55,56]. Although the sequence types associated with these clones are still common culprits in AMX-resistant infections, they are often found with different serotypes due to prevalent capsule switching in the postvaccine era [11,57•]. AMX nonsusceptible clones have also been associated with increased rates of cephalosporin and clindamycin resistance [55].

Serial passaging experiments to select for mutants with reduced susceptibility to β-lactam antibiotics such as piperacillin and cefotaxime provided valuable information regarding antibiotic targets and key PBP affinities for resistance. However, these experiments are challenging to perform for AMX as many residue substitutions are required across multiple loci for detectable changes in resistance [42••,58]. Previous studies have shown no or very minor decreases in susceptibility, with no more than a 2-fold increase in MIC [59]. Maintaining selection for 24 passages did result in a 10-fold increase in MIC, but at 0.125 g/mL was still significantly below the

clinical breakpoint for intermediate resistance [60], and even starting from PEN-resistant clinical isolates did not result in an increase in MIC [61].

Resistant variants primarily arise through homologous recombination among strains, or from closely related species that occupy the upper respiratory tract niche [62]. The result is frequent and large-scale recombination events, enabling an ever-changing landscape of vaccine-escape and antibiotic-resistant variants [63]. β-lactamase enzymes have never been isolated from *S. pneumoniae*. Instead, resistance in this species is predominantly mediated by significant amino acid substitutions in the PBPs, although mutations in non-PBP genes such as *murM* and *ciaH* have also been implicated [40,64,65]. While highly conserved in β-lactam-sensitive strains, PBPs of resistant isolates are enormously variable [40].

Indeed, among AMX-resistant pneumococcal isolates, the relative horizontal transfer frequencies of *pbp2x* and *pbp2b* were high (10.2% and 7.8%, respectively), indicating that movement of *pbp* alleles coding for proteins with low AMX affinity between pneumococcal strains has contributed to the dissemination of resistance [56]. Evidence of *pbp* allele transfer among *Streptococcus mitis*, *Streptococcus oralis*, *Streptococcus gordonii*, and *S. pneumoniae* has been found in clinical isolates [66,67•], while *pbp* alleles from *S. mitis* coding for low-affinity PBPs conferred resistance to PEN and cefotaxime in *S. pneumoniae* under laboratory conditions [68]. β-lactam-susceptible commensal Streptococcal species display large variation in *pbp* alleles as well as regions homologous to low-affinity mosaic *pbp* fragments in β-lactam-resistant *S. pneumoniae*. This provides a global pool of low-affinity *pbp* alleles for lateral gene transfer into, and among, pneumococcal isolates, enabling rapid dissemination of *pbp*-mediated resistance [69,70].

Natural competence and homologous recombination are powerful processes that enable enormous genetic variation and genomic plasticity. Competence is controlled by a quorum sensing pathway that rapidly expresses all proteins necessary for exogenous DNA uptake and fratricide. Upon cytoplasmic entry, ssDNA is bound by RecA, DprA, and SsbB, and the homology search is initiated [3]. Once RecA-mediated crossover has begun, the stringent homology requirement decreases, allowing some mismatches to occur, a requirement for the recombination of highly variable *pbp* loci [71].

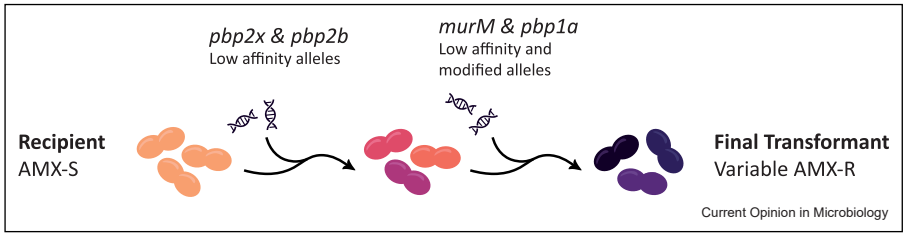
Recombination regions up to 50-Kb long were identified in clinically relevant pneumococcal isolates such as pneumococcal molecular epidemiology network clone 1 (PMEN-1) [63,72]. Events of this length enable simultaneous capsular serotype and resistance profile switching due to the close proximity of the *cps* locus to *pbp2x* and *pbp1a* [73]. In addition, frequent cotransfer of distantly located *pbp* loci provides strong evidence for epistasis between resistance determinants [74,75].

Pbp2x, Pbp2b, Pbp1a, and MurM implicated in amoxicillin resistance

β-lactam resistance in *S. pneumoniae* is mediated by large-scale modifications to the PBPs that reduce the antibiotic-binding affinity. However, no single mutation or block of mutations in any one *pbp* was sufficient to confer AMX resistance in a susceptible strain [76] and multiple residue sites in all three of Pbp1a, 2x, and 2b were found to be under positive selection in resistant isolates [77]. Adding to this complexity are the risks associated with altering the active sites of essential enzymes, requiring compensatory mechanisms to balance fitness loss with antibiotic avoidance. This results in selection for mutations outside of the target loci, as well as a tendency toward an optimal order of resistance determinant acquisition.

Low-affinity alleles of *pbp2x*, *pbp2b*, *murM*, and *pbp1a* were essential to recapitulate the AMX MIC of a resistant serotype 11A isolate (clonal complex 156) in susceptible strains D39V and TIGR4. In concordance with previous work, Pbp2x and Pbp2b substitutions were critical first steps toward resistance [42••,58]. MurM and Pbp1a substitutions were acquired in a second round of transformation, and were essential for

Figure 3



Optimal order of allele uptake for the development of AMX resistance. Acquiring low-affinity alleles of the essential PBPs, Pbp2b and Pbp2x, was demonstrated by both whole genome and polymerase chain reaction (PCR) fragment transformation experiments to be the first key step in AMX resistance development [42••,58]. High-level resistance could then be acquired through the uptake of pbp1a and murM alleles.

high-level AMX resistance (Fig. 3). The TP active-site modifications found in PBPs of resistant pneumococcal isolates can have severe deleterious effects on the cell [78,79]. As such, acquiring determinants in this optimal order facilitates rapid evolution of high-level resistance through fitness compensation.

To further complicate our understanding of AMX resistance in the pneumococcus, genomic context has an enormous effect on the MICs conferred by resistance determinants. In one study, strains carrying identical *pbp* and *murM* alleles displayed different AMX MICs and transforming these alleles into a susceptible lab strain did not confer the same level of AMX resistance as the donor [80]. In a different collection of AMX-resistant clinical isolates, the TP domain of *pbp1a* did not contain mutations, but novel substitutions in Pbp2x were identified [10]. This indicates strongly the existence of more than one molecular mechanism for AMX resistance, but more detailed investigations into AMX-resistant isolates from different lineages is required to explore this idea further.

β-lactam antibiotics differ in their TP active-site interactions, thus substitutions reducing binding affinity to one drug may have an inverse effect on that of another [7]. Despite this, there are many well-described PBP substitutions found in β-lactam-resistant isolates, particularly at the catalytic motifs within the TP domains, which have been thoroughly reviewed previously [40]. In the context of AMX resistance, the active-site serines of Pbp2x include S337, S395, and S571, which have been shown to stabilize cefotaxime embedded in the active site [81], and the respective neighboring sites 338, 394, and 572 were found to be under positive selection in the context of AMX resistance [77]. Outside of the TP domain, relatively few substitutions have been linked to resistance (although many studies only report the sequence of TP domains), and were not found to be essential for AMX resistance [42••]. Within Pbp2b, the T446A substitution has been selected for in laboratory experiments with both piperacillin and AMX [82] (Paddy Gibson, PhD thesis, University of Lausanne, 2022), as well as found in collections of PEN and AMX-resistant clinical isolates [83,84]. A set of 10 mutations at the C-terminus of the Pbp2b TP domain is strongly associated with high-level AMX resistance [77,84]

Mosaic *pbp1a* alleles restore deleterious growth defects conferred by low-affinity *pbp2x* and *pbp2b* alleles [78,85]. In addition, functional Pbp1a is required for expression of the resistance phenotype [42••]. Found in all β-lactam-resistant isolates, the TSQF(574–577)NTGY substitution contributes to a narrower active site [86], while there is evidence for positive selection of S351A and E512K in AMX resistance [77]. In contrast to Pbp2x and Pbp2b, substitutions outside the TP domain of Pbp1a are found in resistant isolates and have been associated with differences in AMX MIC, although the role of these changes in the β-lactam–PBP interaction is not known [77].

Penicillin versus amoxicillin: a changing selective landscape

AMX began to be recommended for clinical use as rates of PEN resistance in *S. pneumoniae* increased. Consequently, AMX resistance evolved when alleles for low β-lactam-binding affinity PBPs had already been circulating in the clinic for more than 20 years, since the first PEN-resistant strain was identified. Despite this, it was initially found that most PEN-resistant isolates could still be treated with AMX, with AMX MICs lower than those for PEN [87,88]. Importantly, this observation implies that resistant mutations acquired under PEN-selective pressure do not necessarily confer the same reduction in PBP affinity for AMX. This is interesting given the close evolutionary relationship between AMX- and PEN- resistant clones, and the strong indications that high AMX MICs evolved within classical PEN-resistant lineages [12,54–56]. Several studies comparing *pbp* allele sequences or restriction fragment length polymorphism patterning within PEN-resistant strains with varying AMX susceptibilities found large variation within *pbp2x* and *pbp1a* [12,58,76,80]. The only PBP substitutions shared among AMX-resistant isolates were in Pbp2b, specifically those located toward the C-terminal end of the TP domain (amino acids 590–641). Up to 10 substitutions have been identified in this region, with AMX MIC increasing with the number of modifications [76]. Indeed, transfer of this block of mutations was critical to reach donor-level AMX MIC in genomic DNA transformation experiments [42••]. Interestingly, this block of mutations could not be transformed into a susceptible lab strain, unless low-affinity alleles of other *pbp* genes and a mutated *murM* allele were already present in the genome [42••,84], potentially explaining the greater ease with which PEN-resistant clones acquired AMX resistance [12].

Close association between *pbp2b* and *murM* alleles in amoxicillin resistance

The presence of modified *murM* alleles results in higher proportions of branched muropeptides in the cell wall and are thought to compensate for losses in PBP fitness [64,89]. Wild-type *murM* is not essential under normal conditions, but deletion of this gene results in almost complete loss of PEN resistance, regardless of any low-affinity PBPs present [90]. The effect of *murM* mutants on the final MIC highly depends on the overall genomic context. For example, transfer of *murM* from a serotype 23F isolate to susceptible lab strain R6 carrying *pbp2x*, *pbp2b*, and *pbp1a* alleles from the same donor was unable to confer comparable resistance. However, the same

murM allele could confer further resistance in R6 when in the presence of *pbp* alleles from different multidrug-resistant clinical isolates [58]. The importance of branched muropeptides to β-lactam resistance is not well understood, although it has been hypothesized that the different lipid-II shape either fits the low-affinity variant active sites better, thus compensating for reduced transpeptidation efficiency, or is a stronger competitor against β-lactams for PBP binding [40].

β-lactam resistance-associated *murM* alleles have been observed to co-occur with specific *pbp2b* mutations in AMX-resistant clinical isolates, including the 590–641 block mentioned above [11,58,76,84]. This suggests an inclination toward branched muropeptides in cell elongation, the major function of Pbp2b. Importantly, Pbp2b-depleted cells have been shown to incorporate more branched muropeptides into the cell wall [23•]. In addition, deleting *murM* introduces synthetic lethality with *pbp2b*, where decreasing Pbp2b concentration becomes toxic in a dose-dependent manner [23•]. This is not the case for Pbp2x, supporting an increased propensity for branched muropeptides in peripheral but not septal cell wall synthesis. One proposed hypothesis is that indirect cross-linking by the longer and more flexible branched muropeptides in glycan strands strengthens the PG structure when cross-linking activity is reduced, and that Pbp2b specificity could thus be due to the increased turgor pressure experienced during division by new PG at the periphery but not the septum [23•].

Outside the penicillin-binding proteins: other determinants implicated in amoxicillin resistance

Less commonly in pneumococcal β-lactam resistance, genetic determinants outside the *pbp* and *murM* loci are implicated. This has not been well studied in the context of AMX resistance, and no AMX-specific determinants have been identified. Nevertheless, PG N-acetylglucosamine deacetylase encoding *pgdA* was first associated with β-lactam resistance in strains with higher AMX than PEN MICs. It was strongly associated with specific *pbp2x* mutations and required to recapitulate both the AMX and PEN MICs of the donor strain following the transformation of genomic DNA from a serotype 19A-resistant clinical isolate into susceptible strain R6 [91].

Selecting for mutants that grew following exposure to AMX identified the two-component signal transduction system CiaRH as a low-level resistance determinant (Paddy Gibson, PhD thesis, University of Lausanne, 2022). Substitutions in the histidine kinase CiaH were first identified following serial passage in cefotaxime and piperacillin, usually clustered proximal to the conserved histidine residue (H226) [65], while more distally locating substitutions have been identified in clinical isolates [92]. CiaRH acts as a general response to cell wall stress and regulates transcription of 21 operons, including those involved in choline uptake and metabolism, TA synthesis, and competence [93,94]. It is not known how CiaH senses cell wall stress, and environment-dependent differences in the response make it challenging to study. Exactly which member(s) of its 21-operon regulon is responsible for the changes in β-lactam susceptibility remains elusive, and it is unlikely to be specific to AMX.

Future outlook

Despite 40 years of clinical use, AMX resistance in the pneumococcus remains low, providing a unique opportunity to act in advance of the problem.

A complex resistance genotype reliant on recombination is challenging to investigate at the bench as the process of horizontal gene transfer itself plays such a critical role in the evolution. Truly reproducing the clinical environment is difficult. Early work with PCR products limited experiments to known resistance determinants [58], and while transformation experiments with isolated genomic DNA expand the determinants that can be studied, it still fails to truly mimic the native environment [42••,91]. We would expect extracellular DNA to be either highly fragmented, or for transfer to occur following lysis of a neighboring cell in close contact, both of which will affect the quantity and quality of DNA taken up [95]. Recent work exploring the effects of cell-to-cell contact on recombination and resistance determinant transfer has perhaps shown the most accurate experimental design for these contexts and may provide a platform for future work on AMX resistance evolution [96]. Finally, the selection of donor and recipient strains is perhaps the most engineered aspect of these experiments. We use highly resistant donors from the clinic but transform their DNA into fully susceptible laboratory strains that have not been found in patients in more than 70 years. To fully discern how AMX resistance has developed, and is currently evolving, it is important to consider the serotypes and sequence types represented in AMX- resistant isolates. Utilizing recipient strains already encoding reduced susceptibility to PEN and perhaps other drugs is critical, as the genomic context even outside the PBPs will play a role both in recombination, and in how protein modifications are tolerated by the cell.

One approach to slow the evolution of antibiotic resistance in *S. pneumoniae* is to inhibit natural competence and thus reduce horizontal gene transfer. Conveniently, a side effect of this approach is attenuated virulence, as murein hydrolases such as LytA and CbpD are

upregulated during competence, increasing adherence to host cells as well as triggering the release of cytoplasmic pneumolysin [37,97•]. Competence-stimulating peptide (CSP) analogs block competence quorum sensing, inhibiting the resulting transcriptional cascade [97•,98]. In addition, proton motive force blockers, such as the biocide triclosan, have been shown to disrupt competence onset and effectively reduce horizontal gene transfer both in vitro and in an in vivo mouse model [99]. Long-term evolutionary effects at the population level of these types of cotherapeutics have yet to be explored.

In the search for cotherapeutic molecules that increase the killing potential of a drug while reducing the likelihood of resistance development, the use of high-throughput screening to assess bacterial stress response on the whole-genome level is being widely applied [100•,101]. Indeed, sCrilecs-seq identified the mevalonate pathway as a potential cotherapeutic target. Inhibiting isoprenoid synthesis with the FDA-approved drug clomiphene (Fig. 1), in combination with AMX, was able to resensitize an AMX-resistant pneumococcal isolate [30]. This work emphasizes the tight associations between cell wall synthesis pathways, which can be exploited for the development of cotherapeutics, and highlights the future role of chemogenomics in ongoing research to combat the antibiotic resistance pandemic.

In conclusion, the complex evolutionary trajectories and delicate protein interactions that comprise β-lactam resistance in *S. pneumoniae* offer many challenges for the study of AMX resistance. However, they also present many potential routes for the inhibition of resistance development and the treatment of AMX-resistant pneumococcal infections.

Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

No data were used for the research described in the article.

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