Pseudomonas aeruginosa: targeting cell-wall metabolism for new antibacterial discovery and development

Pseudomonas aeruginosa is a leading cause of hospital-acquired infections and is resistant to most antibiotics. With therapeutic options against *P. aeruginosa* dwindling, and the lack of new antibiotics in advanced developmental stages, strategies for preserving the effectiveness of current antibiotics are urgently required. β-Lactam antibiotics are important agents for treating *P. aeruginosa* infections, thus, adjuvants that potentiate the activity of these compounds are desirable for extending their lifespan while new antibiotics – or antibiotic classes – are discovered and developed. In this review, we discuss recent research that has identified exploitable targets of cell-wall metabolism for the design and development of compounds that hinder resistance and potentiate the activity of antipseudomonal β-lactams.

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The discovery of antibiotics was one of the greatest advancements in modern medicine and paved the way for now-routine surgeries and other invasive medical procedures. Multidrug-resistant bacteria, however, are a looming threat to human health, complicating the safety of treatments that we currently take for granted. Among the most prominent multi-drug resistant bacteria is *Pseudomonas aeruginosa* – a Gram-negative pathogen and frequent cause of healthcare-associated infections, including surgical-site infections, urinary tract infections, bloodstream infections and ventilator-associated pneumonia. Collectively, *P. aeruginosa* is responsible for ~13% of all serious healthcare-associated infections and ~440 deaths in the USA each year [1]. *P. aeruginosa* also causes a variety of community-acquired infections, including contact lens keratitis, ear infections in small children and lung infections in cystic fibrosis patients, among others.

P. aeruginosa is an opportunistic pathogen notable for its high levels of intrinsic antibiotic resistance, bolstered by its ability to rapidly adapt to myriad environmental stresses [2]. In recognition of its role as an emerging public health threat, *P. aeruginosa* was included in the 'most-wanted' list of ESKAPE pathogens (with *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanii* and *Enterobacter* spp.) for which treatment options are dwindling [3]. Thus, new antibiotics or antibiotic adjuvants that potentiate or rescue currently available drugs are urgently required for the treatment of *P. aeruginosa* infections [3,4].

In this review, we provide an overview of the primary resistance mechanisms in *P. aeruginosa* and detail potential targets that may be exploitable for combination therapies. Because the discovery of new antibiotics or antibiotic classes lags the need for new entities, we suggest that strategies that preserve the effectiveness of current antibiotics are among the most direct approaches for fighting *P. aeruginosa* infections. Given the long-

term success of antipseudomonal antibiotics that target cell–wall biosynthesis, we emphasize potential avenues by which the lifespan of those drugs can be extended.

Mechanisms of antibiotic resistance in *P. aeruginosa*

Intrinsic resistance

P. aeruginosa has low-level intrinsic resistance to most classes of antibiotics, primarily due to its relatively impermeable outer membrane (OM) barrier that limits the penetration of drugs, coupled with chromosomally encoded multidrug efflux pumps that extrude drugs from the cell. An inducible AmpC β-lactamase that degrades common β-lactam antibiotics also contributes significantly to resistance (Table 1).

The OM of *P. aeruginosa* is its first defense against antibiotics and other harmful compounds. Small hydrophilic molecules, including β-lactams, tetracyclines and fluoroquinolones access the cell through OM porins (OMPs), while hydrophobic drugs, including macrolides, aminoglycosides and cationic peptides access the periplasm by diffusion and self-promoted uptake [24,25]. The permeability of *P. aeruginosa*'s OM is approximately 1–10% that of *E. coli* due to multiple factors, but particularly the limited diffusion of antibiotics due to OMPs with small diffusion pores [5,26] (Table 1). The overall number of general diffusion porins is also lower in *P. aeruginosa* than *Enterobacteriaceae* since *P. aeruginosa* preferentially acquires nutrients through dedicated porins [25]. The size exclusion limit of OMPs in *P. aeruginosa* approaches that of the size of small antibiotics, slowing passage of drugs into the cell [27]. The anionic drugs carbenicillin, azlocillin and piperacillin are either larger than the OMP's constriction zone or make poor critical contacts within the channel and thus cross ineffectively [28]. Conversely, zwitterionic drugs, including ampicillin and amoxicillin form stronger bonds within OMPs (particularly at the constriction site), which facilitates passage [28,29].

The entry of hydrophobic antibiotics is limited by their diffusion across the OM, an asymmetric structure with the inner leaflet comprised of phospholipids and the outer leaflet of lipopolysaccharides (LPS) (Table 1). The lipid A and inner core heptose regions of *P. aeruginosa* LPS are primarily responsible for resisting the passage of antibiotics and are stabilized by divalent cations that cross-bridge adjacent LPS molecules [30]. The resulting reduction in the net negative charge on the OM leads to more closely packed LPS and reduces the passage of small molecules. The lipid A moiety of LPS can undergo aminoarabinosylation, limiting its interactions with positively charged antibiotics and antimicrobial peptides [31]. Collectively, these features limit OM permeability.

Upon entering the periplasm, antibiotics encounter additional intrinsic resistance mechanisms, including multiple multidrug efflux pumps and the chromosomally encoded AmpC β-lactamase (Table 1). Combined with reduced diffusion across the OM, these periplasmic mechanisms can efficiently eliminate the antibiotic [2]. Genes encoding efflux pumps and β-lactamases were likely acquired by horizontal gene transfer. However, because they have become incorporated into the *P. aeruginosa* chromosome, and are present in all *P. aeruginosa* isolates, they are considered intrinsic resistance factors [32].

Efflux-mediated antibiotic resistance in *P. aeruginosa* is conferred primarily by the tripartite pumps of the resistance/nodulation/division superfamily, which extrude multiple classes of antibiotics and other substrates. The primary resistance/nodulation/division efflux pumps influencing antibiotic resistance are MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM [6], whose substrates include (but are not limited to) β-lactams, fluoroquinolones and aminoglycosides [27]. The loss of efflux in clinical strains with high-level fluoroquinolone or β-lactam resistance causes hypersensitivity, and efflux pump inhibitors have been explored as potential therapeutic options. Plaguing their therapeutic development, however, has been their ancillary cyto- and nephrotoxicity [33,34].

β-lactam antibiotics are among the most widely used drugs for the treatment of *P. aeruginosa* infections; however, many are degraded by the inducible AmpC β-lactamase. AmpC is an Ambler class C cephalosporinase [35] that inactivates common antipseudomonal β-lactam antibiotics, including piperacillin, cefotaxime and ceftazidime [15]. Due to the effectiveness of β-lactams in treating *P. aeruginosa* infections, combination therapies with β-lactamase inhibitors are common in clinical usage. For example, combinations of piperacillin or ceftolozane and the β-lactamase inhibitor tazobactam are used to treat critically ill patients [36,37]; however, resistance to such therapies – though less likely – has been observed [38,39].

Acquired resistance

Intrinsic mechanisms alone are sufficient to confer antibiotic resistance in *P. aeruginosa*; however, cells can become more resistant by the acquisition of transferrable genetic elements that carry resistance genes, or through the accumulation of mutations that bolster intrinsic mechanisms [2] (Table 1). Among the primary resistance genes acquired by way of transferrable genetic elements are those encoding aminoglycoside-modifying enzymes (AMEs), which modify aminoglycosides to reduce their affinity for the 30S ribosomal subunit, and β-lactamases that hydrolyze

Table 1. Mechanisms of antibiotic resistance in *Pseudomonas aeruginosa*.

Class	Mechanism	Effect	Examples of key factors involved
Intrinsic	OM impermeability	Inherently low drug penetrance	Genes involved in LPS biosynthesis (e.g., *lpt, wbp*) [2]; low-level OMP expression and OMPs with small diffusion pores [5]
	Multidrug efflux pumps	Constitutive low-level extrusion of small molecules	*mexAB, oprM* [6]
	AmpC β-lactamase	β-lactam hydrolysis	*ampC* [7]
Acquired	OM impermeability	Modified porin structure or expression; reduced drug penetrance	Mutations in *oprD* [8,9], acquisition of *mcr-1*[†] [10]
	Multidrug efflux pumps	Increased drug efflux	Mutations in *nfxB* [11], *nfxC* [12], *mexR* [13]
	AmpC β-lactamase	High-level AmpC expression and β-lactam resistance	Mutations in *dacB, ampD* [14] or *ampR* [15]
	Horizontal acquisition of other resistance genes	Modification of antibiotics or their targets (e.g., AMEs) or β-lactamases)	AMEs: AAC(3′), AAC(6′); Methylation of 16S rRNA: RmtABCD; β-lactamases: TEM, CTX, SHV, IMP, VIM, NDM-1 [16]
Adaptive	OM impermeability	Reduced drug penetrance	*oprDGI* [17,18], *arnABCDE* [19]
	Multidrug efflux pumps	Increased drug efflux	*mexXY, oprM,* PA5471 [20]
	AmpC β-lactamase	AmpC induction	Increase in 1,6-anhydroMurNAc-peptide production [21]; BlrAB TCS [14]
	Biofilm formation	Slow metabolic activity, antibiotic inaccessibility, increased drug efflux	PhoPQ TCS [2], *pmr* operon [22], *blrD* [23]

AME: Aminoglycoside-modifying enzyme; CTX: Cefotaxime hydrolyzing; IMP: Imipenem hydrolyzing; LPS: Lipopolysaccharide; OM: Outer membrane; OMP: Outer membrane protein; TEM: Temoniera; SHV: Sulfhydryl variable; VIM: Verona integron-encoded metallo-β-lactamase.
[†]The *mcr-1*-containing plasmid has not yet been isolated from *P. aeruginosa*; however, transfer between other Gram-negatives has been reported [10].

β-lactams [2]. When combined with the activity of the AmpC β-lactamase encoded on the *P. aeruginosa* chromosome, acquired genetic elements encoding extended-spectrum β-lactamases [40] and metallo-β-lactamases [41,42] substantially add to the range of β-lactams that *P. aeruginosa* can resist – leaving few treatment options for such strains [43].

Acquired resistance also occurs from the accumulation of mutations that facilitate the survival of *P. aeruginosa* in the presence of specific antibiotics. In most cases, mutational resistance occurs upon treatment with sublethal concentrations of antibiotics, and a single mutation can confer high-level resistance [14,44]. However, the accumulation of multiple mutations – each of which confers small increases in resistance – can lead to high-level resistance in a stepwise manner [2,45–48]. While most studies focus on single mutations that confer high-level resistance, recent work from our laboratory [46,47] and others [44,49], confirm that high-level resistance is achievable by the accumulation of multiple mutations that alone confer low level resistance. This phenomenon has been referred to as 'creeping baselines' [48]; however, the frequency of such a phenomenon is difficult to assess in clinical isolates because creeping baselines are likely to be missed by most clinical microbiology procedures that focus on defined break points [27].

The frequency of mutations that lead to antibiotic resistance can be influenced by the specific antibiotic, and mode of growth (e.g., mutation frequency during biofilm growth is greater than during planktonic growth) [50]. Notable resistance mutations that have been observed in clinical multidrug-resistant *P. aeruginosa* isolates occur in genes that encode repressors of efflux pumps. Loss-of-function mutations in *mexR* [13], *nfxB* [11] or *nfxC* [12] lead to an increase in expression of the MexAB-OprM, MexCD-OprJ and MexEF-OprN efflux pumps, respectively, conferring resistance to β-lactams, fluoroquinolones and aminoglycosides.

Of particular interest for the treatment of *P. aeruginosa* infections are resistance mutations conferring high-level β-lactam resistance. In most cases, such mutations lead to increased expression of AmpC or an increase in efflux pump activity by the mechanisms described above. Constitutive overexpression of AmpC is caused by loss-of-function mutations in *dacB,* which encodes the low-molecular-weight (LMW) penicillin-binding protein (PBP) PBP4, or in *ampD*, which encodes the cytosolic amidase, AmpD [14,51]. The steps leading to the induction of AmpC are dif-

ferent between *dacB* and *ampD* mutants, and despite being incompletely defined, it is clear that loss of these enzymes alters peptidoglycan (PG) metabolism, leading to an increase in AmpC expression that is dependent on the transcriptional regulator, AmpR. One notable exception to the AmpR-AmpC paradigm of β-lactam resistance concerns resistance toward the carbapenem, imipenem, which is poorly hydrolyzed by AmpC. Imipenem is not a substrate of known efflux pumps, and resistance is primarily conferred by changes to the porin, OprD, through which imipenem enters the cell. Reductions in *oprD* expression, or structural changes that constrict the OprD channel, lead to imipenem resistance [8,9].

Adaptive resistance

In addition to its repertoire of intrinsic resistance mechanisms, *P. aeruginosa* can rapidly alter gene expression profiles in response to diverse environmental stimuli, including exposure to antibiotics (Table 1). This form of antibiotic resistance – called adaptive resistance – is dependent upon continued exposure to sublethal concentrations of drug, since resistance profiles revert upon removal of the stimulus [27]. Because of the transient nature of adaptive resistance mechanisms, they have remained largely unexplored. In general, adaptive resistance occurs when transcriptional or translational changes transiently bolster intrinsic resistance mechanisms [2,48].

Among a multitude of responses, adaptive resistance often involves the transitory upregulation of multidrug efflux pumps. Such an adaptive mechanism can occur upon treatment of *P. aeruginosa* with aminoglycosides; the reactive oxygen species generated induce the expression of *mexXY-oprM* via PA5471, increasing resistance [20] (Table 1). The precise mechanisms surrounding this response, and the role of PA5471 remain incompletely understood; however, it is a specific response toward drugs that target the ribosome [20].

Treatment with sublethal concentrations of antibiotics results in global changes to gene expression profiles. For example, sublethal concentrations of ceftazidime and ciprofloxacin induce the expression of genes involved in the SOS response – including *dinP*, which encodes the error-prone DNA polymerase IV that increases mutational frequency – and antibiotic resistance genes encoding multidrug efflux pumps and AmpC [17,18]. Treatment of *P. aeruginosa* with these drugs also caused downregulation of the OMPs, OprG, OprI and OprD, decreasing OM permeability [17,18].

Multiple two-component systems (TCSs) are responsible for regulating *P. aeruginosa* gene expression in response to antibiotic treatment. Exposure to antimicrobial peptides leads to the activation of the PhoPQ, ParRS and CprRS TCSs (among others), which causes global gene expression changes. These include upregulation of the *arn* operon that leads to aminoarabinose modification of the lipid portion of the LPS, increased expression of efflux pumps, reduced expression of *oprD* and increased biofilm formation [19,52–55]. Treatment with the β-lactams imipenem or cefoxitin activate the BlrAB (formerly CreBC) TCS that contributes to AmpC expression [23], while aminoglycoside exposure activates the AmgRS TCS that is thought to respond to cytoplasmic membrane perturbations due to the incorporation of mistranslated proteins [56]. Activation of AmgRS induces the expression of multiple factors, including the membrane protease HtpX that degrades misfolded proteins released from dysfunctional ribosomes [57].

Among the key responses to sublethal antibiotic treatment is the formation of biofilms, which are more tolerant of antibiotics and disinfectants than their planktonic counterparts [58] (Table 1). Mechanisms of biofilm tolerance include the overexpression of efflux pumps, LPS modification in cells of the upper biofilm layers [22,27], plus reduced growth rates and formation of persister cells, limited drug penetrance through densely organized biofilms, and/or antibiotic trapping by a charged extracellular polymeric substance matrix [59]. Nearly all classes of antibiotics stimulate *P. aeruginosa* biofilm formation at subinhibitory concentrations, including aminoglycosides [58,60,61], β-lactams [23,62], fluoroquinolones [60], macrolides [63] and tetracyclines [60], suggesting it is an early nonspecific response to mitigate damage until other, class-specific resistance mechanisms are activated.

Quorum sensing (QS) also influences biofilm formation and antibiotic resistance in *P. aeruginosa*. *P. aeruginosa* has two QS systems – *las* and *rhl* – that sense and respond to diffusible small molecules, which accumulate in a population-dependent manner and induce biofilm formation [64]. However, QS circuits in *P. aeruginosa* are also activated in response to environmental stresses – independent of population density – indicating that this system also plays a role in the adaptive promotion of biofilms [65]. Moreover, one QS signaling molecule, *Pseudomonas* quinolone signal (PQS) precursor, 4-hydroxy-2-heptylquinoline (HHS), is a substrate for *mexEF-oprN* [66], whose expression is adapted under antibiotic challenge and may play a role in biofilm promotion by increasing extracellular concentrations of HHS. Notably, *mexEF-oprN* expression is controlled by the global regulator MvaT, which also regulates QS circuitry, virulence factor expression and biofilm formation [67].

Cell-wall metabolism & AmpC β-lactamase induction

Perhaps the best-studied resistance response to β-lactam challenge is the inducible expression of AmpC. AmpC induction is primarily an adaptive response to β-lactam challenge; however, specific mutations that alter PG metabolism cause constitutive AmpC expression and high-level β-lactam resistance. Mutations in *dacB* and *ampD* – which encode the LMW PBP4 and the cytosolic amidase AmpD, respectively – confer high-level β-lactam resistance in many clinical isolates.

Like most bacteria, *P. aeruginosa* cells are surrounded by a mesh-like polymer called PG that protects them from high internal turgor pressures and maintains their structural integrity. PG is composed of alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) sugars that are cross-linked through stem peptides attached to MurNAc [68]. Maintenance and turnover of PG to allow growth and division of the cell requires PBPs, amidases, endopeptidases, carboxypeptidases and lytic transglycosylases (LTs). High-molecular-weight PBPs (e.g., PBP1) have both transglycosylase and transpeptidase activities responsible for linking GlcNAc and MurNAc sugars, and cross-linking stem peptides, respectively, while others (PBP2, 3) have transpeptidase activities only. LMW PBPs (i.e., PBP4, 5, 6, 7) have endopeptidase and carboxypeptidase activities, responsible for cleaving stem peptides to control the extent of crosslinking within PG [68]. Cell growth and division require PG turnover, during which LTs cleave the PG backbone between GlcNAc and MurNAc residues, yielding anhydromuropeptides (anhMP). Under normal conditions, most anhMP enter the cytoplasm through a permease, AmpG and are efficiently processed by enzymes including the β-*N*-acetylglucosaminidase, NagZ and AmpD, for recycling into *de novo* PG biosynthesis (Figure 1). If anhMP that enter the cytoplasm are not processed (as is the case in an *ampD* mutant), they accumulate, bind to AmpR – thereby displacing the UDP-MurNAc-pentapeptides that otherwise maintain AmpR in a repressor conformation – and it then activates AmpC expression (Figure 1) [7,69].

Treatment with β-lactam antibiotics blocks PG transpeptidation, leading to an imbalance between the insertion of new material and lysis of the existing PG. The imbalance between synthetic and lytic activities increases the amount of anhMP generated, leading to AmpC expression [7,70]. This process can occur even in the presence of functional AmpD, as the increased accumulation of cytoplasmic anhMP quickly saturates AmpD activity (Figure 1). While *ampD* mutations occur in the clinic, leading to β-lactam resistance, mutations in the carboxypeptidase PBP4 are more frequently observed [14]. The specific mechanism underlying this efficient, one-step conversion to high-level β-lactam resistance in *P. aeruginosa* is not known; however, studies in *Aeromonas* spp. suggest that AmpC induction is caused by the increased accumulation of a specific anhMP (anhydromuro-pentapeptide) [71]. AmpC expression in *Aeromonas* spp. is positively regulated by a TCS called BlrAB (Blr: β-lactam resistance) rather than by an AmpR-mediated pathway, as in *P. aeruginosa*. Interestingly, *P. aeruginosa* has a homologous BlrAB (formerly CreBC) TCS [71] implicated in β-lactam resistance. BlrAB is activated by PBP4-targeting β-lactams, or by PBP4 loss-of-function mutations in the absence of drug [14]. Upon activation, the sensor kinase, BlrB, phosphorylates the response regulator, BlrA, a positive regulator of BlrD (formerly CreD) expression. BlrD is a membrane protein of unknown function with homology to colicin-resistance proteins and is upregulated in response to β-lactam treatment; however, its deletion has little effect on resistance [14]. BlrAB is activated in strains lacking PBP4. Interestingly, disruption of BlrAB in the PBP4 mutant causes a pronounced reduction in β-lactam resistance; however, high AmpC levels remain unchanged [14].

Combination therapies for antibiotic potentiation

Since the golden era of antibiotic discovery from the 1940s to 1960s [72], resistance to all antibiotic classes has occurred, while few new classes have been described. Thus, multiple approaches are necessary for the development of new therapeutic options. Such approaches include the discovery of new classes of antibiotics, but also the identification of adjuvants that could be used in combination to potentiate the activity of current antibiotics [73,74]. Such combination approaches have been exploited for decades to treat cancers, HIV, tuberculosis and other bacterial infections. In fact, some of the best-known antibacterial combination therapies combine β-lactam antibiotics with β-lactamase inhibitors. Piperacillin/tazobactam (i.e., Tazocin™ or Zosyn™, Pfizer Inc., NY, USA) combines a β-lactamase inhibitor with β-lactam antibiotic for treatment of infections caused by *P. aeruginosa* and other pathogens. Although not necessarily for the treatment of *P. aeruginosa* infections, other combination therapies have included trimethoprim/sulfamethoxazole (generic Bactrim™), which inhibits folate synthesis, and quinupristin/dalfopristin (Synercid®, Pfizer Inc., NY, USA), which inhibits protein synthesis. Both compounds included in these combinations are antibiotics themselves that inhibit different enzymatic steps in the same pathway; however, approaches to potentiating antibiotics include an adjuvant that alone may or may not have antibiotic activity, but potentiates the activity of the primary antibiotic. In the case of piperacillin/tazobactam, where tazobactam alone

has no antibacterial activity; however, it inhibits the primary mechanism of resistance against piperacillin–β-lactamase activity. Combinatorial approaches where each drug acts by different mechanisms may also be efficacious for potentiating current antibiotics. For example, the impermeability of *P. aeruginosa* and other Gram-negative pathogens is a formidable obstacle for many antibiotics; however, OM permeabilizers are promising for the treatment of such infections. Such a strategy has been used recently to sensitize Gram-negative pathogens to multiple classes of antibiotics, including the bulky antibiotics daptomycin, vancomycin, teicoplanin and telavancin, which are normally incapable of entering the cell [46,75–78]. Thus, combination therapies hold great promise for rescuing the activity of common antibiotic classes – or previously abandoned scaffolds – and may be useful for extending the lifespan of current antibiotics while advances are made in other areas of antibacterial discovery and development.

The majority of antipseudomonal antibiotics in current clinical use target cell–wall biosynthesis. Thus, we focus here on targets that we feel are exploitable for the development of other cell wall synthesis inhibitors, or cell wall-related adjuvants. In most cases, the targets yet to be explored will allow for the preservation of β-lactams; however, we also discuss potential avenues to increase the potency of other antibiotic classes by increasing membrane permeability.

Untapped targets for adjuvant development

Lytic transglycosylases

The activity of lytic transglycosylases (LTs) is essential for AmpC induction [7] since the inducing molecule is a 1,6-anhydroMurNAc-peptide formed only by their actions (Figure 1). Studies in *E. coli* showed that loss of LT activity prevented AmpC induction; however, a recent study from our laboratory showed that *P. aeruginosa* strains lacking up to five of the ten known LTs

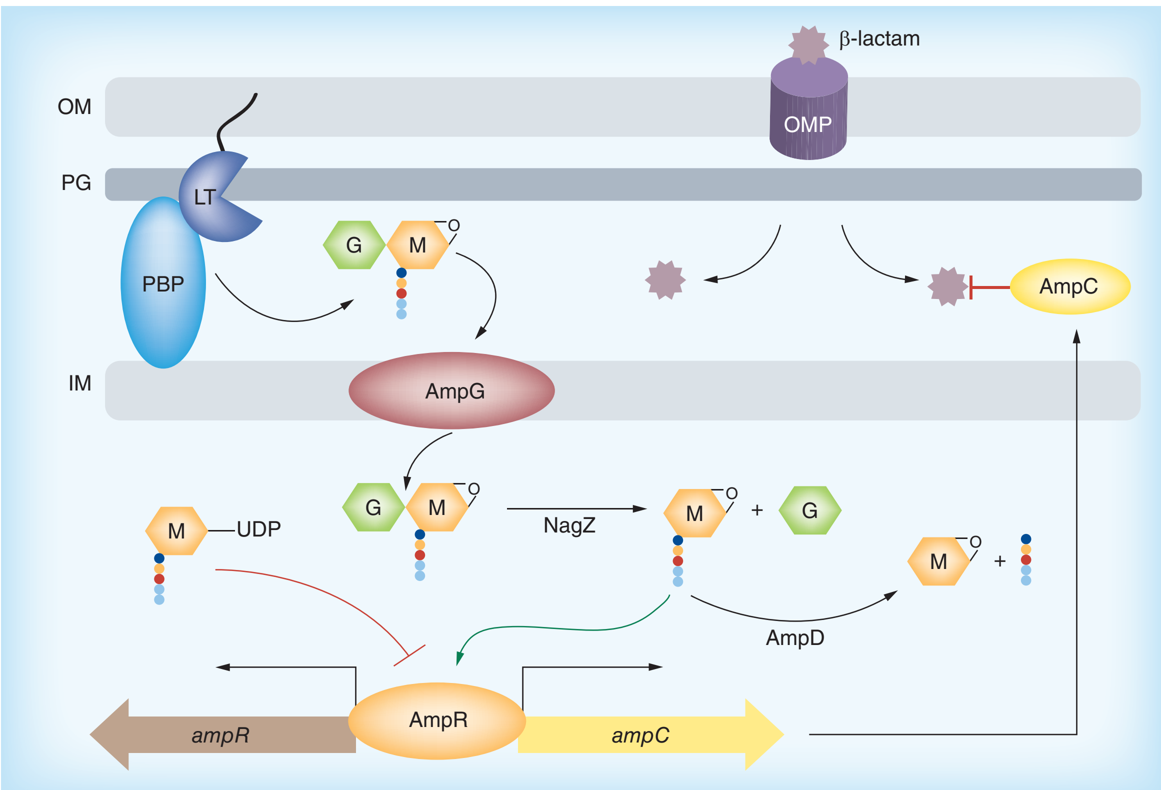

Figure 1. Peptidoglycan-recycling pathway in *Pseudomonas aeruginosa*. Under basal conditions, anhydromuropeptides (anhMPs) are released from the PG layer into the periplasm by the actions of LTs and PBPs. AnhMPs enter the cytosol through the permease, AmpG, are processed by the β-*N*-acetylglucosaminidase, NagZ and the amidase, AmpD, for recycling back into the growing PG layer. Under basal conditions, AmpR is bound by UDP-MurNAc-pentapeptides, repressing AmpC expression. When cytosolic levels of 1,6-anhydroMurNAc-peptides increase – due to β-lactam challenge or inactivation of PBP4 or AmpD – those metabolites bind AmpR, displace the repressing ligands and convert AmpR into an activator of AmpC expression. AmpC translocates to the periplasm where it hydrolyzes β-lactams. Amino acid sequence of the pentapeptide attached to the MurNAc, L-Ala-D-Glu-meso-Diaminopimelic acid-D-Ala-D-Ala.
G: GlcNAc; IM: Inner membrane; LT: Lytic transglycosylase; M: MurNAc; OM: Outer membrane; PBP: Penicillin-binding protein; PG: Peptidoglycan.

continued to exhibit robust induction [46]. That study also unexpectedly revealed that the membrane-bound LTs (mLTs) were required for maintaining the structural integrity of the OM in *P. aeruginosa*. Loss of multiple mLTs led to increased OM permeability and sensitized the cells to β-lactams, bile salts and the bulky glycopeptide antibiotic, vancomycin, which is normally ineffective against *P. aeruginosa*. Importantly, it was the physical presence of the mLTs – but not their enzymatic function – that was required for maintaining OM integrity, as strains expressing inactive mLTs had wild-type resistance profiles [46]. As therapeutic advancements are made to prevent the expression of specific proteins [79] or induce their destabilization and premature degradation, it is foreseeable that mLTs will be of interest as their loss caused antibiotic hypersensitivity. Previous studies have been limited, in that the full complement of LTs have not been deleted; however, given the essentiality of LTs for PG remodeling, pan-inhibitors of LT activity may themselves be antibacterial or potentiate the activities of β-lactams.

The major soluble LT, Slt, is emerging as a preferred LT target for antibiotic adjuvants. Seminal work from Holtje and colleagues [80] revealed that the Slt inhibitor, bulgecin (Table 2), repressed AmpC expression in *E. coli*, suggesting that LT inhibitors may potentiate the activity of penicillins and cephalosporins against AmpC-expressing pathogens. More recently, a study by Cho *et al.* [81] revealed that loss of Slt in *E. coli* caused β-lactam sensitivity, hypothesized to be due to the loss of its role as a quality control enzyme that – upon β-lactam challenge – prevented the aberrant and thus lethal incorporation of PG precursors into the growing PG layer. Our studies in *P. aeruginosa* [46,47] revealed that loss of Slt activity caused hypersensitivity to β-lactams, but not due to decreased AmpC expression. It is unknown whether the same mechanisms lead to increased β-lactam sensitivity of *P. aeruginosa* and *E. coli slt* mutants; however, inhibition of Slt may be useful for potentiation of β-lactam antibiotics in these and other Gram-negative species.

The permease, AmpG

PG metabolites released into the periplasm by the action of LTs are translocated to the cytoplasm via an inner membrane permease, AmpG [7] (Figure 1). A second putative permease homolog, AmpP (PA4218), has also been described in *P. aeruginosa*; however, its loss did not affect resistance to the β-lactams ampicillin, amoxicillin, cefotaxime, ceftazidime or to imipenem [95,96], even though AmpP inactivation caused an ~80–85% reduction in basal and induced AmpC expression [95].

AmpG is a 14-transmembrane domain cytoplasmic membrane protein [95] of the major facilitator superfamily [97]. Studies of *E. coli* AmpG revealed that its preferred substrate is the GlcNAc-anhydroMurNAc disaccharide, and that transport was not influenced by the presence or length of the stem peptide [93]. Loss of AmpG prevents the entry of PG metabolites into the cytosol, and thereby prevents AmpC induction in *P. aeruginosa* [98]. In clinical isolates with constitutive AmpC expression – due to mutations in *dacB* or *ampD,* or overexpression of efflux pumps – and pan-β-lactam resistance, loss of AmpG prevented AmpC expression and caused hypersensitivity to β-lactam antibiotics [98]. Thus, compounds that block AmpG function have promise as adjuvants to protect β-lactam antibiotics.

AmpG belongs to the oligosaccharide-H^+ symporter family [97] and substrate translocation is driven by the energy derived from proton motive force. Cells treated with carbonylcyanide m-chlorophenylhydrazone (Table 2) failed to transport PG fragments [93], which caused β-lactam hypersensitivity in laboratory and clinical strains of *P. aeruginosa* [96,98]. While carbonylcyanide m-chlorophenylhydrazone also likely disrupted the function of multidrug efflux pumps, thus contributing to the increase in β-lactam sensitivity, modulators of proton motive force are attractive on multiple fronts since they would simultaneously inhibit numerous energetic processes [99].

NagZ

PG metabolites enter the cytoplasm via AmpG as GlcNAc-1,6-anhydroMurNAc-peptides [100]. In the cytosol, the β-(1,4)-glycosidic bond between the nonreducing GlcNAc and the 1,6-anhydroMurNAc-peptide is cleaved by the β-*N*-acetylglucosaminidase, NagZ [101,102] (Figure 1). Loss of NagZ prevents formation of the AmpC-inducing 1,6-anhydroMurNAc-peptide and attenuates AmpC expression and β-lactam resistance in strains harboring *dacB* or *ampD* mutations [103,104].

Previous studies aimed at developing NagZ inhibitors focused on derivatives of the β-glucosaminidase inhibitor *O*-(2-acetamido-2-deoxy-D-glucopyranosylidene) amino N-phenylcarbamate (PUGNAc) (Table 2) [94,105–106]. Such compounds are not themselves antibacterial; however, treatment of *P. aeruginosa* strains lacking *ampD* – which leads to high-level AmpC production – with *O*-(2-deoxy-2-*N*-2-ethyl-butyryl-D-glucopyranosylidene)amino *N*-phenylcarbamate (EtBuPUG) reduced ceftazidime and aztreonam minimum inhibitory concentrations (MICs) [103]. However, the lack of specificity for PUGNAc derivatives for NagZ versus related human enzymes has hampered their development for therapeutic purposes [105]. Recently, Stubbs *et al.* synthesized a series of N-acyl analogues of 2-acetamido-2-deoxynojirimycin and identified multiple compounds that more potently

Table 2. Common inhibitors of cell wall metabolism in *Pseudomonas aeruginosa*.

Target	Common inhibitors and inhibitor scaffolds	Effect of inhibition	Structures of common inhibitors and inhibitor scaffolds
Transpeptidases	β-lactams [82]	Loss of PG crosslinking	Piperacillin:
Transglycosylases	Moenomycins [83]; dalbavancin [84]	Loss of PG polymerization	Moenomycin:
Lipid II	Nisin [85], vancomycin [85], tryptamines [86]; telavancin [87]; ramoplanin [85]; other lantibiotics [85]; teixobactin [88]	Loss of PG polymerization	Nisin:

†CCCP is not a direct inhibitor of AmpG, but rather, prevents its function by disrupting proton motive force.
CCCP: Carbonylcyanide m-chlorophenylhydrazone; PG: Peptidoglycan.

Table 2. Common inhibitors of cell wall metabolism in *Pseudomonas aeruginosa*.

Target	Common inhibitors and inhibitor scaffolds	Effect of inhibition	Structures of common inhibitors and inhibitor scaffolds
Lytic transglycosylases	Bulgecin [80]; *N*-acetylglucosamine thiazoline [89]; iminosaccharides [90]; Ivy [91]; hexa-*N*-acetylchitohexaose [92]	Loss of GlcNAc-1,6-anhydroMurNAc production	**Bulgecin:**
AmpG	†CCCP [93]	Prevents transport of GlcNAc-1,6-anhydroMurNAc from the periplasm into the cytosol	**CCCP:**
NagZ	PUGNAc [94]; EtBuPUG [94]; iminosaccharides [90]	Prevents 1,6-anhydroMurNAc monosaccharide production	**PUGNAc:**

†CCCP is not a direct inhibitor of AmpG, but rather, prevents its function by disrupting proton motive force.
CCCP: Carbonylcyanide m-chlorophenylhydrazone; PG: Peptidoglycan.

inhibited NagZ (Table 2) compared with related human enzymes and increased the sensitivity of *P. aeruginosa* to multiple β-lactam antibiotics [105]. One such inhibitor (at the relative high concentration of 1 mM), 2-butamido-1,5-imino-1,2,5-trideoxy-D-glucitol, caused a fourfold decrease in ceftazidime, ampicillin and cefoxitin MICs. Whether *P. aeruginosa* can develop resistance to such inhibitors remains to be assessed; however, if development of resistance reduces the affinity of NagZ for its GlcNAc-1,6-anhydroMurNAc-peptide substrate, its function would effectively be compromised. Thus, either scenario would be expected to attenuate AmpC induction [70].

AmpR

AmpR is a LysR-type transcription factor and a global regulator in *P. aeruginosa* [107]. The *ampR* and *ampC* genes are expressed from a divergent promoter, where AmpR regulates the expression of both genes (Figure 1) [108]. Under baseline conditions, AmpR is a homotetramer bound to UDP-MurNAc-pentapeptide precursors and acts as a repressor of AmpC expression [69]. Upon β-lactam challenge, the cytosolic accumulation of 1,6-anydroMurNAc-peptides saturates the amidase, AmpD and the unprocessed metabolites (with either tri- or penta-peptide stems) bind AmpR. The 1,6-anhydroMurNAc-peptides displace UDP-MurNAc-pentapeptide, converting AmpR into an activator of AmpC expression [21,109] (Figure 1). Without AmpR, cells fail to express AmpC upon treatment with an inducing β-lactam (e.g., cefoxitin) – even in *dacB* clinical isolates that otherwise have constitutive AmpC expression – restoring β-lactam sensitivity [14]. The crystal structure of AmpR's effector-binding domain (EBD) – in apo- and repressor-bound states – was recently solved, and critical residues required for AmpC induction identified [69,110]. Those studies also shed light onto the conformational changes that AmpR may undergo upon repressor binding [69], facilitating design of allosteric inhibitors that could prevent DNA binding, or trap AmpR in the repressor state. Similarly, inhibition of AmpR–DNA interactions, or AmpR tetramer formation, may potentiate the activity of current β-lactam antibiotics by blocking AmpC induction.

The BlrAB TCS

Among the TCSs that control responses to the ever-changing environmental conditions to which *P. aeruginosa* must adapt [111] is the BlrAB TCS, implicated in β-lactam resistance [14]. Treatment with cefoxitin (a PBP4 inhibitor), or mutations in *dacB,* activate BlrAB, causing a subsequent increase in the expression of BlrD, an inner membrane protein of unknown function. Whether BrlD has a role in β-lactam resistance is unclear, as loss of this protein caused only marginal reductions in β-lactam MICs in a clinical *P. aeruginosa* isolate with a loss-of-function mutation in *dacB*. However, *blrD* mutants grew more slowly and had reduced fitness in *in vitro* competition experiments [23]. Conversely, loss of BlrAB in the same *dacB* mutant caused four- and eight-fold reductions in ceftazidime and piperacillin MICs, respectively [14]. The ligand for the sensor kinase, BlrB, has not been identified in *P. aeruginosa*; however, studies in *Aeromonas hydrophila* suggest that it is GlcNAc-1,6-anhydroMurNAc-pentapeptide [71]. Consistent with such a metabolite being the *P. aeruginosa* ligand is the finding that BlrAB is primarily activated upon loss or inhibition of PBP4 [14,23], which causes a 1.5-fold increase in metabolites containing pentapeptides [112]. Moreover, the simultaneous inactivation of the LMW PBPs 4 and 5 – both D, D-carboxypeptidases – leads to an ~18-fold increase in metabolites containing pentapeptides and ~10-fold higher *ampC* expression than that of the strain lacking PBP4 alone [112].

Loss of BlrAB also reduced the rate of mutations leading to ceftazidime resistance in *P. aeruginosa* [14]. High-level ceftazidime-resistant mutants arose from wild-type PAO1 at 3×10^{-8} per cell division, while strains lacking BlrAB developed resistance at a rate of $<1 \times 10^{-11}$ (the detection limit). From those data, it was suggested that the BlrAB TCS contributes to development of β-lactam resistance in *P. aeruginosa* [14].

Inhibitors of TCSs have previously been discovered using a combination of high-throughput screening, and structure-based and rational design [113]. Histidine kinases are attractive targets since loss of catalysis, ATP-binding or dimerization each prevents signal transduction. However, these features are conserved across all HKs making the targeting of specific enzymes difficult [114]. With the acquisition of additional crystal structure information for HKs, structure-based rational design approaches will enable the development of inhibitors with higher specificities [114]. Arguably, a lack of specificity may be a benefit for such inhibitors since, in addition to having broad-spectrum activity across multiple species, they would likely inhibit multiple targets within the same cell.

LytC-type PG amidases

LytC-type *N*-acetylmuramyl-L-alanine amidases cleave the bond between MurNAc and the first L-alanine on the stem peptide of PG [115], and their activity is essential for growth and division in *P. aeruginosa* [116]. *P. aeruginosa* has two such amidases, AmiA and AmiB [117], with AmiA being expendable while loss of AmiB causes cell separation defects and eventual cell death [116]. Moreover, loss of AmiB also increased OM permeability and caused hypersensitivity to gentami-

cin and vancomycin, and reversed imipenem resistance arising from *oprD* mutations. Thus, inhibitors of AmiB may not only lead to lethal growth defects, but also increase OM permeability, thereby promoting their own uptake.

Since the uncontrolled activities of amidases may cause aberrant PG cleavage or cleavage at nonseptal sites, activators of AmiB also have potential as antibiotic adjuvants in *P. aeruginosa*. As in *E. coli* [118,119], LytM (lysostaphin/peptidase M23)-domain-containing factors are also likely required for stimulating the activity of AmiB in *P. aeruginosa* [116]. *P. aeruginosa* encodes three LytM proteins, NlpD, NlcS and EnvC, whose collective inactivation was lethal, while single and double mutants were viable. Based on those data, it was hypothesized that each LytM factor can activate AmiB. Consistent with this suggestion, the loss of LytM factors also sensitized cells to vancomycin [116].

A complicating factor for targeting AmiB – or its activators – with antibiotic adjuvants is that suppressor mutations in the *cpxA* gene reversed the lethal phenotype associated with loss of AmiB, and restored OM integrity [116]. Whether such suppressors invoke a Cpx-like stress response [120], similar to that observed in *E. coli*, and whether that will hinder the development of antibiotic adjuvants targeting AmiB will need to be addressed [116].

Peptidoglycan transglycosylases

PG synthesis requires transglycosylase (TG) and transpeptidase (TP) activities. *P. aeruginosa* has two bi-functional Class A PBPs – PBP1a and PBP1b – whose TG activities are responsible for polymerizing the growing PG layer. The TP activities of PBP1a and PBP1b, as well as those of the Class B PBPs 2 and 3, crosslink adjacent stem peptides to give the PG layer strength and rigidity. The activity of at least one Class A PBP is required for survival, as the loss of one enzyme is compensated for by the TG activity of the other. PBPs are excellent antibiotic targets – as evidenced by the widespread and long-term use of β-lactams – and inhibition of TG activity provides a promising, yet underutilized avenue for new antibacterial development [121].

Perhaps the best-studied TG inhibitor is moenomycin A (Table 2); however, the therapeutic utility of this and related compounds is hampered by their poor pharmacokinetic properties [122]. Moreover, moenomycin-family antibiotics are generally ineffective against Gram-negatives due to their inability to cross the OM [123]. Recognizing this problem, recent studies have increased efforts to discover LMW compounds with TG-inhibitory activity [86,121] (Table 2). To this end, a series of tryptamine-based inhibitors were recently found to inhibit growth of *E. coli* at concentrations as low as 8 μg/ml [86]. Unlike moenomycins which are structurally similar to lipid II and directly bind bifunctional PBPs [83], the tryptamine-based compounds appear to a have a similar mechanism of action to nisin, vancomycin and bacitracin, interacting with lipid II to prevent PG biosynthesis [86]. Similarly, the novel antibiotic, teixobactin, recently discovered in a screen of previously unculturable bacteria, inhibited cell-wall biosynthesis by interacting with lipid II [88] (Table 2). Teixobactin was ineffective against wild type *P. aeruginosa* and *E. coli*; however, an *E. coli* strain with a defective OM had an MIC of 2.5 μg/ml, suggesting that OM-permeabilizing adjuvants may increase the potency of teixobactin against Gram-negatives, including *P. aeruginosa*.

Combinations with OM permeabilizers

The utility of many otherwise effective antibiotics (e.g., glycopeptides) is hampered by their inability to access intracellular targets in *P. aeruginosa*. One approach to overcome this problem is therapeutic combinations in which one of the compounds is an OM permeabilizer. Often, OM permeabilizers have cytotoxic side effects on eukaryotic cells; however, with a lack of new antibiotics in the developmental pipeline – and the increasing prevalence of antibiotic-resistant infections – the use of such compounds may become a necessity. For example, colistin (polymyxin E) has recently been reintroduced into clinics as a drug of last resort for treating infections caused by multi-drug-resistant Gram-negative pathogens, including *P. aeruginosa* [124]. Colistin was removed from clinical use in the 1970s due to concerns over the potential for nephrotoxicity and neurotoxicity [125]; however, the lack of effective therapeutic options made its revival unavoidable. In *P. aeruginosa*, multiple studies have shown combination therapies involving colistin and other antibiotics, including β-lactams, fluoroquinolones, aminoglycosides and rifampin to be synergistic against clinical isolates [126–129]. Colistin resistance has been observed in *P. aeruginosa* for decades due to mutational and adaptive mechanisms [130]; however, mutational resistance imparts a fitness cost and is not horizontally transmissible while adaptive resistance is transient and reverts upon removal of the drug [10,131]. Recently, the startling (and unpleasant) discovery that the *mcr-1* gene conferring colistin resistance is encoded on a transmissible plasmid that has spread to multiple countries [10] suggested that the long-term utility of colistin–drug combinations is questionable.

Due to the potential for OM permeabilizers to significantly enhance the potency of multiple classes of antibiotics, recent studies have identified additional

non-toxic compounds that synergize with other antibiotics by increasing OM permeability [132,133]. Two lactoferricin (cationic pepsin cleavage product of lactoferrin) derivatives, P2-15 and P2-27, increased the potency of multiple antibiotic classes against *P. aeruginosa* strains. These compounds resensitized a carbapenem-resistant strain lacking OprD to imipenem, and fluoroquinolone-resistant clinical isolates overexpressing efflux pumps to ciprofloxacin [133]. Another study showed that polyethylenimine – a nontoxic synthetic polycationic polymer – synergized with ceftazidime and the Gram-positive antibiotic, novobiocin, against *P. aeruginosa* [132]. Thus, with further medicinal chemistry investigations, there is strong potential for the discovery of additional nontoxic OM permeabilizers that potentiate the activities of multiple classes of antibiotics against *P. aeruginosa*, including those not generally used against Gram-negative pathogens.

Dual-action compounds where one of the actions is increased permeability have been described as promising therapeutic agents, as they inhibit key intracellular functions and also promote their own uptake. The efflux pump inhibitor phenylalanine-arginine-β-naphthylamide (PAβN) inhibits the MexAB-OprM, MexCD-OprJ and MexEF-OprN efflux pumps in *P. aeruginosa*, and sensitizes cells to β-lactams and fluoroquinolones [134]. This compound permeabilizes the OM of *P. aeruginosa* and sensitizes to the glycopeptide vancomycin that is neither a known substrate of efflux pumps nor typically active against *P. aeruginosa* because of its inability to cross the OM [75]. Thus, the dual action of PAβN – and compounds like it – is an asset for combination therapies, increasing the potency of other antibiotics.

Dual-action lantibiotics permeabilize the *P. aeruginosa* OM and have intracellular antibacterial targets. Nisin is the best example of such compounds; it forms pores in the OM – due to its activity as a cationic peptide – and inhibits PG synthesis by interacting with lipid II [135]. Nisin and another lantibiotic with a similar mode of action, lacticin 3147 [136], has been shown to exhibit synergistic antipseudomonal activities when combined with polymyxin E and clarithromycin [137]. Multiple modes of action are becoming increasingly observed for many antimicrobial peptides [138], and when combined with conventional antibiotics, they could have profound effects against Gram-negative pathogens, including *P. aeruginosa*.

Other strategies that promote the uptake of antibiotics include those involving so-called Trojan horses [139]. The Trojan horse strategy exploits nutrient uptake systems to effectively smuggle antibiotics into the cell. Iron is an essential nutrient, and bacteria use siderophores to acquire it from the environment – where it is typically in very limited supply [140]. The Trojan horse strategy has been effective against *P. aeruginosa,* where tris-catecholate siderophores conjugated to the β-lactams ampicillin or amoxicillin reduced MICs from >100 to 0.39 μM [141]. Conjugation of ampicillin to the *P. aeruginosa* siderophore, pyoveridin, also led to hypersensitivity of an otherwise resistant PAO1 strain [142]. Supporting the utility of such approaches, the investigational antibiotic BAL30072 is currently in Phase I clinical trials [143]. BAL30072 is a monobactam derivative conjugated with an iron-chelating dihydroxypyridone moiety. BAL30072 has activity against multiple Gram-negative pathogens, including a *P. aeruginosa* strain (MIC_{90} = 8 μg/ml) that was resistant to aztreonam, imipenem, ceftazidime and piperacillin-tazobactam (MIC_{90} >32 μg/ml for all) [144]. Thus, by coupling antibiotics to substrates for nutrient uptake systems, the primary mechanism of intrinsic resistance – that is, OM impermeability – can be circumvented to decrease effective antibiotic concentrations.

Conclusion & future perspective

The increasing prevalence of multidrug-resistant *P. aeruginosa* is a global concern, which has been further elevated with the discovery that resistance toward the last-resort antibiotic, colistin, is transferrable between bacteria. Fortunately however, many promising avenues remain to be exploited for the preservation of conventional antipseudomonal antibiotics. In most cases, at least some inhibitors of the targets discussed here have been identified and can be used as a starting point for medicinal chemistry investigations. As other ways to facilitate the transfer of antibiotics across the OM are discovered, it is foreseeable that many antibiotics that have historically been used only against Gram-positive pathogens could be deployed against *P. aeruginosa*.

It should be mentioned that the untapped targets discussed here are not without their own obstacles. One notable caveat is the necessity for antibiotic adjuvants to access the cytoplasm, which will require bypassing the OM impermeability barrier and efflux mechanisms. The potential for resistance toward such compounds will also have to be evaluated.

It is foreseeable that over the next decade the arsenal of antibiotics available for the treatment of *P. aeruginosa* infections will expand while maintaining the utility of conventional antibiotics. Headway is being made in overcoming many of the hurdles that previously hampered therapeutic discovery and development – including the inability to culture potentially rich sources of untapped natural prod-

ucts or due to the limitations associated with the design of synthetic compounds [72], among others – which is expected to result in additional lead compounds making it to clinical trials. Due in part to the expansion of compound libraries, and improvements to structure-based rational design strategies, it is expected that the next 5–10 years will see increases in the number of lead compounds, chemical scaffolds and targets that can be exploited for therapeutic purposes. Previous target-based approaches have been hampered by the lack of substrates or biochemical intermediates with which to evaluate the inhibitory activities of hit compounds. Advances in the synthesis of such substrates (e.g., PG intermediates), or the development of assays with which to evaluate the inhibition of key physiological processes (e.g., *in vitro* PG biosynthesis assays) will likely lead to the resurrection of promising targets and compounds that had previously been discarded due to the lack of these developmental tools [145]. The growing and widespread recognition *P. aeruginosa* as a major public health burden will continue to drive the efforts to develop new therapeutic approaches against this pathogen.

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Executive summary

The problem

- *Pseudomonas aeruginosa* is a frequent cause of hospital-acquired infections and is resistant to nearly all antibiotics.
- β-Lactam antibiotics are the primary therapeutic agents against *P. aeruginosa*; however, expression of AmpC β-lactamase, reduced outer membrane permeability and efficient multidrug efflux pumps hamper their utility.
- No new antibiotics or antibiotic classes are expected to market in the near future.

Therapeutic strategies to help manage the problem

- Factors involved in peptidoglycan metabolism that prevent the induction of AmpC expression are promising targets for the potentiation and preservation of β-lactam antibiotics, and may be useful as drug targets themselves.
- Combination therapies with outer membrane permeabilizers hold potential for sensitizing *P. aeruginosa* to current antibiotics and those that are generally only active against Gram-positive bacteria.

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