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REVIEW

An Interplay of Multiple Positive and Negative Factors Governs Methicillin Resistance in *Staphylococcus aureus*

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SUMMARY The development of resistance to β-lactam antibiotics has made *Staphylococcus aureus* a clinical burden on a global scale. MRSA (methicillin-resistant *S. aureus*) is commonly known as a superbug. The ability of MRSA to proliferate in the presence of β-lactams is attributed to the acquisition of *mecA*, which encodes the alternative penicillin binding protein, PBP2A, which is insensitive to the antibiotics. Most MRSA isolates exhibit low-level β-lactam resistance, whereby additional genetic adjustments are required to develop high-level resistance. Although several genetic factors that potentiate or are required for high-level resistance have been identified, how these interact at the mechanistic level has remained elusive. Here, we discuss the development of resistance and assess the role of the associated components in tailoring physiology to accommodate incoming *mecA*.

KEYWORDS *Staphylococcus aureus*, MRSA, MecA, antimicrobial resistance

INTRODUCTION

A dramatic increase in antimicrobial resistance (AMR) among human pathogens has been reported by the World Health Organization (1). This clinical challenge is compounded by the lack of a mechanistic understanding of the emergence of bacterial resistance as well as a decline in new antibiotic discoveries (2). Among human pathogens, *Staphylococcus aureus* is a prominent example of the spectre of AMR (3, 4).

Penicillin was first introduced to treat patients with *S. aureus* bacteremia in the early 1940s, but as early as 1942, the first penicillin-resistant strains of *S. aureus* were isolated (5). The development of penicillin resistance is mediated by an enzyme, penicillinase/β-lactamase, encoded by *blaZ*, which cleaves the β-lactam ring and destroys the action of the antibiotic (4, 6).

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TABLE 1 Genetic factors required for MRSA β-lactam resistance

Gene/operon	SAOUHSC[a]	Functional class	Function	Reference(s)
mecA		Extracellular PG synthesis	Encodes the heterologous penicillin-binding protein PBP2A with low affinity for β-lactam antibiotics	158
mecB		Extracellular PG synthesis	Encodes a heterologous penicillin-binding protein PBP2B with low affinity for β-lactam antibiotics	46
mecC		Extracellular PG synthesis	Encodes a heterologous penicillin-binding protein PBP2C with low affinity for β-lactam antibiotics	41, 42
pbp4	00646	Extracellular PG synthesis	PBP with transpeptidase activity	47

[a]*S. aureus* 8325 genome locus tag.

In response to the spread and emergence of penicillin resistance, a semisynthetic β-lactam named methicillin, impervious to β-lactamase, was developed and introduced into the clinic in 1959 (7, 8). One year later, methicillin-resistant *S. aureus* (MRSA) was recovered from a patient diagnosed with osteomyelitis and septic arthritis (9). Since then, antibiotic resistance in *S. aureus* has spread in epidemic waves, with MRSA initially being largely a nosocomial infection, leading to so-called hospital-associated MRSA (HA-MRSA) (10–13). More recently, community-associated MRSA (CA-MRSA) has emerged as a major clinical threat, creating a reservoir of MRSA in and out of health care settings (10, 11). CA-MRSA can be genetically distinguished from HA-MRSA, also having less of a portfolio of antibiotic resistance properties and often making the toxin Panton-Valentine leukocidin (PVL) (14). However, there are now many examples of how CA-MRSA has spread to health care settings, blurring the distinction between the two MRSA types (11). Additionally, MRSA can be harbored by livestock (livestock-associated MRSA [LA-MRSA]), where it can cause disease in those animals and also spread to humans via contact (15, 16).

MRSA is a clear clinical threat, whereby the outcomes of MRSA-mediated infections are worse than those of methicillin-sensitive *S. aureus* (MSSA) (17). In addition, MRSA means resistance to nearly all available β-lactams (18). Currently, MRSA-mediated infections account for significantly high morbidity and mortality rates (19, 20).

GENETIC FACTORS REQUIRED BY MRSA FOR β-LACTAM RESISTANCE

Penicillin binding proteins (PBPs) are essential for the final stages of bacterial cell wall peptidoglycan (PG) biosynthesis and are the targets of penicillin and other β-lactams (21, 22). Methicillin resistance is, most commonly, mediated by the acquisition of a novel penicillin-binding protein, 2A (PBP2A), which is able to take over the activities of endogenous enzymes (PBPs) due to its low affinity for β-lactam antibiotics (Table 1) (23). Using a penicillin-binding assay, several researchers identified the novel low-affinity PBP2A encoded by *mecA* in MRSA strains (21, 24, 25). It was confirmed that *mec* is found only on the chromosome of MRSA and not MSSA and is explicitly required for resistance (26). The *mecA* gene and regulatory elements are encoded on a mobile genetic element found in all MRSA strains called the staphylococcal cassette chromosome (SCC*mec*), which has been acquired from an exogenous, environmental source (27). The degree of expression of methicillin resistance is determined by *mecI* and *mecR1* (28), regulatory elements encoded within the *mec* gene complex adjacent to *mecA* on the chromosomal SCC*mec* element (29). The regulatory genes *mecR1* and *mecI* are structurally and functionally similar to the *blaZ* regulatory components *blaR1* and *blaI*, which, in response to β-lactam antibiotics, induce the expression of *mecA* and *blaZ*, respectively (8, 30). It is important to note that some MRSA strains carry a modified version of the *mec* regulatory system that may lack *mecI* or include truncated versions of some genes (31, 32). The strains with the complete regulatory system tend to be slower in the induction of resistance when challenged with methicillin than those with an incomplete system (33).

SCC*mec* elements contain three basic genetic components and share a structurally similar backbone that consists of (i) a *mec* gene complex carrying *mecA* and its regulators, surrounding open reading frames (ORFs), and insertion sequences, (ii) a cassette

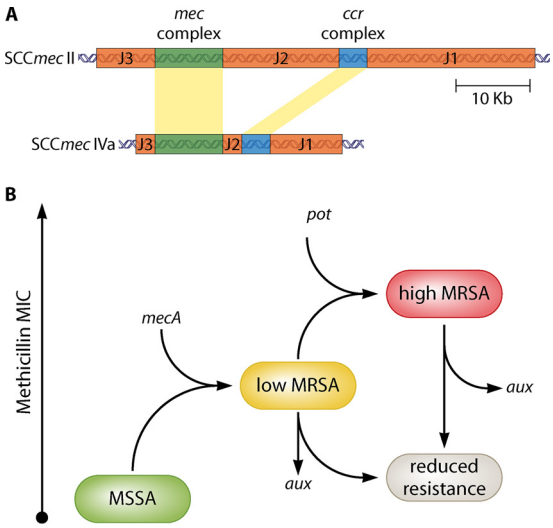

FIG 1 Factors required and associated with β-lactam resistance by MRSA. (A) SCC*mec* element. The major features of SCC*mec* types II and IV are shown as examples of HA-MRSA and CA-MRSA, respectively (based on reference 11). The *mecA* gene is contextualized within SCC*mec* of different sizes, by the cassette chromosome recombinase (*ccr*) gene complex and the joining regions (J1 to J3) that can encode a variety of other functions, including housekeeping and transposons. (B) Roles of Aux and Pot factors. There are two steps required for an MRSA strain to develop high-level resistance to β-lactams. The first is acquisition of the *mecA*/SCC*mec* cassette that is required for resistance *per se*. This, however, provides only the ability to grow in the presence of low concentrations of β-lactams. High-level MRSA is supported by those factors associated with resistance. The second step, leading to high-level resistance, requires a mutation in a potentiator gene (*pot*). Auxiliary factors (*aux*) are required to support resistance.

chromosome recombinase (*ccr*) gene complex containing *ccrAB* and/or *ccrC*, ensuring the mobility of SCC*mec* and surrounding ORFs, and (iii) the joining region (J region) (Fig. 1A) (34). Some SCC*mec* elements also carry housekeeping genes inside J regions as well as transposons (35). Based on the nature of the *ccr* and *mec* gene complexes, coupled with the location and DNA segments of the J regions, SCC*mec* elements are classified into types I to XIII and different subtypes (11, 36, 37). SCC*mec* types I, II, and III are large elements encoding resistance to several classes of antibiotics and have historically been associated with prevalent HA-MRSA clones (10). These include the archetypal strains COL (type I) and N315 (type II) (38). More recently, types IV and V have become more common, associated with CA-MRSA but also some widespread HA-MRSA strains, here again making it more difficult to designate isolates as HA/CA MRSA (39). Strain USA300 (type IV) has been used to generate an ordered transposon mutation library as a community-wide resource (40).

Although different SCC*mec* elements share structural similarities and carry *mecA*, SCC*mec* type XI carries a *mec* homolog classified as *mecC*, originally designated $mecA_{LGA251}$ (Table 1) (41, 42). The *mecC* gene encodes PBP2C, which shares only 63% identity at the amino acid level with PBP2A encoded by *mecA* (41). However, strains carrying *mecC* exhibit methicillin resistance properties similar to phenotypes of strains with *mecA* in a temperature-sensitive manner (43). Also, mutagenesis experiments have confirmed that *mecC* mediates β-lactam resistance in different *S. aureus* strain backgrounds (42). The important difference between the PBPs encoded by *mecC* and *mecA* is that PBP2C has higher binding affinity for oxacillin than cefoxitin in comparison to PBP2A, offering an unusual antibiotic susceptibility profile for these two antibiotics and providing a useful tool to distinguish them (44). Also, in contrast to PBP2A, PBP2C does not require the transglycosylase activity of PBP2 to exhibit high-level oxacillin resistance, indicating that PBP2C might preferentially interact with monofunctional transglycosylase(s) (43). Very recently, *mecC*-derived MRSA has revealed exciting insights into the evolution of antibiotic resistance. Strains of LA-MRSA with *mecC* have been found to have been harbored by hedgehogs in the

preantibiotic era and to have subsequently spread to livestock and humans (45). In the hedgehog reservoir, a dermatophyte fungus has been found to produce β-lactams that create a selective advantage for MRSA strains in the natural host, providing a nice example of the environmental development of antimicrobial resistance (45).

MRSA can also be attained via a plasmid-borne *mecA* homologue called *mecB* (Table 1) (46). Finally, in the laboratory setting, MRSA can be selected for without the need for *mecA*, or its homologues, via mutations associated with the gene encoding the endogenous PBP4 (Table 1) (47). This occurs via missense mutations surrounding the active site with unknown consequences and alterations to the promoter region resulting in overproduction of PBP4 (47).

Borderline oxacillin-resistant *S. aureus* (BORSA) is also clinically relevant (48). BORSA strains do not have *mecA* or *mecC*, demonstrate borderline levels of resistance to β-lactamase-resistant penicillins, and typically hyperproduce β-lactamase, sometimes associated with point mutations in native PBP-encoding genes (48).

GENETIC FACTORS ASSOCIATED WITH β-LACTAM RESISTANCE BY MRSA

Methicillin resistance requires PBP2A to take over all the transpeptidase functions of the endogenous PBPs that are inhibited by the β-lactams. This is an extraordinary feat for an enzyme that has evolved outside *S. aureus*, given the complex interactions that govern peptidoglycan (PG) homeostasis during growth and division (49). Once *mecA* is introduced into the genome of *S. aureus*, PBP2A therefore does not act in isolation from the endogenous systems required for cell growth and division, having a significant impact on cellular physiology (50). Those processes that are needed for cell wall homeostasis require a tight integration of many different factors (51), and thus it is no surprise that, despite being essential for methicillin resistance through its ability to function as an alternative transpeptidase, PBP2A is not the only factor associated with resistance to β-lactams (52). Indeed, early studies performed using transposon mutagenesis allowed the identification of a set of chromosomal genes whose function is required for *mecA*-associated methicillin resistance (51, 53, 54). As these genes were not the mediators of methicillin resistance *per se*, they were named auxiliary (*aux*) genes or factors (Fig. 1B). Here, the genes and proteins that have been published to act as auxiliary factors are highlighted in bold, listed in Table 2, and displayed in Fig. 2. Given the role of Aux factors in supporting the ability of PBP2A to facilitate PG synthesis, their study also sheds light on the interconnectedness of basic physiological processes that underpin growth and division. Many of the Aux group of components are those that provide precursors needed for correct synthesis of the cell wall, but this group also includes factors that are involved in a whole variety of cellular physiological processes, such as the **GlnR** repressor from nitrogen metabolism (55), the acyl carrier protein of fatty acid biosynthesis **HmrB** (56), the surface protein **FmtB**, and **FmtC** (**MprF**), which is required for lysinylation of phosphatidyl glycerol in the cell membrane (57, 58).

Interestingly, the levels of methicillin resistance of most MRSA isolates are not the same even if all Aux factors remain intact. One of the characteristics of MRSA isolates is their ability to express β-lactam resistance in so-called heterogeneous fashion (59). The majority of cells in a bacterial population are resistant only to low concentrations of methicillin ($\leq$5 μg mL^{-1}). However, a small minority of cells (10^{-4} to 10^{-3}) exhibit high-level methicillin resistance ($>$50 μg mL^{-1}). The heterogeneous culture, in turn, has the potential to develop homogeneous resistance, where all cells are uniformly resistant to high concentrations of methicillin (60). This conversion can be induced under laboratory conditions by exposure to β-lactams. Once strains have converted from heterogeneous to high-level homogeneous resistance, this remains even in the absence of antibiotic and is not connected with genetic mutations in *mec* or *aux* genes (61, 62). Constitutive expression of *mecA* does not lead to homogeneous resistance (32, 63). Most of the genes that have been identified to potentiate the conversion to high-level resistance are not directly involved in cell wall biosynthesis or

TABLE 2 Auxiliary (Aux) factors: mutations reduce β-lactam resistance

Gene/operon	SAOUHSC	Functional class	Function	Reference(s)
glmS	02399	Intracellular PG synthesis	Glucosamine-6-phosphate synthase	78
glmM (*femD*)	02405	Intracellular PG synthesis	Phosphoglucosamine mutase	84
murA	01146	Intracellular PG synthesis	Transferase; converts UDP-GlcNAc to UDP-GlcNAc-enoylpyruvate	78
murB	00752	Intracellular PG synthesis	Reductase; converts UDP-GlcNAc-enoylpyruvate to UDP-MurNAc	78
murC	01856	Intracellular PG synthesis	UDP-*N*-acetylmuramate-L-alanine ligase	78
murD	01147	Intracellular PG synthesis	UDP-*N*-acetylmuramoylalanine-D-glutamate ligase	78
murE	00954	Intracellular PG synthesis	Catalyses incorporation of lysine into the peptide stem	78, 159
murF	02317	Intracellular PG synthesis	UDP-*N*-acetylmuramoyl-tripeptide-D-alanyl-D-alanine ligase	78
femX (*fmhB*)	02527	Intracellular PG synthesis	Addition of the first glycine to the peptide stem	78, 81
femA	01373	Intracellular PG synthesis	Addition of the 2nd and 3rd glycine to the peptide stem	82
femB	01374	Intracellular PG synthesis	Addition of the 4th and 5th glycine to the peptide stem	83
murT	02107	Intracellular PG synthesis	Mur ligase homolog	78, 160
glyS	01666	Intracellular PG synthesis	Glycine tRNA synthetase	78
murJ	01871	Extracellular PG synthesis	Lipid II translocase	161
pbp1 (*pbpA*)	01145	Extracellular PG synthesis	PBP with transpeptidase activity	78
pbp2	01467	Extracellular PG synthesis	PBP with transpeptidase and transglycosylase activity	89
pbp4	00646	Extracellular PG synthesis	PBP with transpeptidase activity	90
ftsW	01063	Extracellular PG synthesis	Transglycosylase	78
tarO	00762	Cell wall synthesis	Forms 1st precursor in WTA synthesis	162
tarA	00640	Cell wall synthesis	Forms 2nd intermediate in WTA synthesis	52
tarB	00643	Cell wall synthesis	Forms 3rd intermediate in WTA synthesis	52
tarD	00645	Cell wall synthesis	Involved in WTA synthesis	52
tarL	00227	Cell wall synthesis	Polyribitol-phosphate extension of WTA	78
tarI	00225	Cell wall synthesis	WTA synthesis	52
tarS	00228	Cell wall synthesis	Glycosyltransferase	92
ltaS	00728	Cell wall synthesis	Lipoteichoic acid synthase	54
fmtB (*mrp*)	02404	Cell wall synthesis	Cell surface protein	57
ftsA	01149	Cell division	Divisome component	78
ftsZ	01150	Cell division	Divisome component	78
glnR	01285	Regulation and cell signaling	Glutamine synthetase repressor	55
fmtA	00998	Regulation and cell signaling	Membrane protein	58
fmtC (*mprF*)	01359	Regulation and cell signaling	Lysinylation of membrane phosphatidylglycerol	58
prsS	00200	Regulation and cell signaling	ECF sigma factor	116
sigB	02298	Regulation and cell signaling	Transcription factor	112
sarA	00620	Regulation and cell signaling	Accessory regulator A	114
pknB	01187	Regulation and cell signaling	Eukaryotic-like serine/threonine kinase	54, 114
vraSR	02098/9	Regulation and cell signaling	Two-component signal transduction sensor of cell wall stress	99
spsB	00903	Protein secretion	Signal peptidase I	78
prsA	01972	Protein folding and stabilization	Required for posttranslational maturation of PBP2A	106
htrA1	01838	Protein folding and stabilization	Required for posttranslational maturation of PBP2A, acts in synergy with PrsA	107
hmrA	02374	Protein stability	Endopeptidase	56, 117
hmrB	01201	Metabolism	Homologue of acyl carrier protein	56
gatD	02106	Metabolism	Glutamine amidotransferase	160
sucC	01216	Posttranslational modification	β subunit of succinyl-coenzyme A synthetase	97
sucD	01218	Pos-translational modification	α subunit of succinyl-coenzyme A synthetase	97
cycA	01803	Transport	Putative amino acid permease gene	74, 163
auxA	01025	Hypothetical protein	Putative transmembrane transporter protein	96
auxB	01050	Hypothetical protein	Putative transmembrane protein	96

linked to PBP2A. In many cases, they are responsible for regulation of cell physiology in the broader sense (50, 59, 64, 65). Genome sequencing of laboratory-derived *mecA*-containing mutants selected on high concentrations of methicillin (100 μg mL^{-1}) has revealed that mutations in several genes can lead to high-level methicillin resistance (66). The genes in which mutations lead to increased β-lactam resistance we have called "potentiators" (*pot*), as opposed to auxiliary genes (*aux*), in which mutation leads to decreased resistance (Fig. 1B). The Pot factors are therefore both intriguing and perplexing as to how point mutations in genes not apparently linked to PBP2A function can tailor physiology and result in such a profound effect on resistance levels.

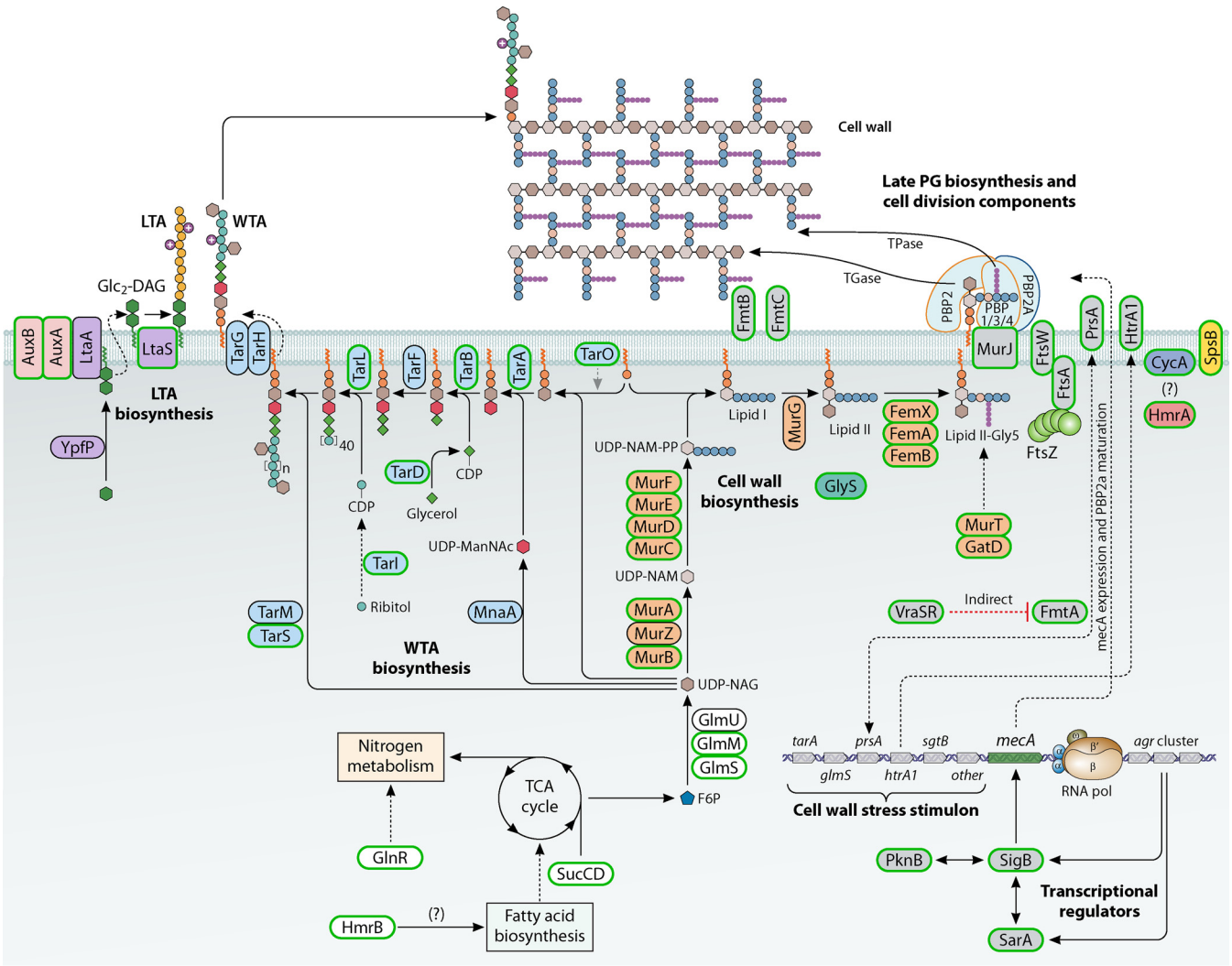

FIG 2 Schematic model of the range of Aux factors involved in β-lactam resistance by MRSA. The Aux factors form a body of components largely involved in cell wall homeostasis and the response to stress. Auxiliary factors have a green outline, and those with a black outline have no demonstrated impact on methicillin resistance. Factors involved in the same biological pathway have a common shading color. TCA, tricarboxylic acid.

Given the wide range of SCC*mec* types (11) and clonal lineages (67, 68), it is likely that some of the *aux* and *pot* factors may be strain specific. High-level MRSA can be established in the laboratory using just *mecA* in an MSSA background (50, 69), suggesting that there are factors independent of SCC*mec*. Therefore, without systematic analysis across a broad range of backgrounds, the role of SCC*mec* cannot be verified. With that caveat, we have covered the portfolio of factors in order to begin to establish those common, underlying mechanisms that underpin the development and maintenance of resistance in MRSA.

Thus, high-level MRSA involves an underlying prerequisite for an exogenous PBP, set within the genetic and physiological context of an MSSA cell, where Aux and Pot factors provide a yin and yang of resistance capabilities.

Auxiliary (Aux) Factors: Mutations Reduce β-Lactam Resistance

Aux factors (Fig. 2; Table 2) support resistance by ensuring that PBP2A is able to carry out cell wall synthesis in the presence of antibiotics. Mutation of any of the Aux factors upsets the delicate framework that allows this to occur. It is currently unknown if PBP2A becomes part of the integrated cell growth and division machinery, which involves multiple protein-protein interactions (70, 71), or acts more independently. The presence of *mecA* in cells, in the absence of antibiotic stress, leads to a raft of

transcriptional changes with consequential metabolic outputs (50). This suggests that having the active enzyme itself creates a stress on the cells, which have to alter their physiology to accommodate this. The Aux factors can therefore be categorized into three major groups: those are directly involved in cell wall homeostasis, those that control it (particularly in response to cell wall stress), and components involved in more general stress responses required to ensure cellular fitness in changing environments.

Cell wall homeostasis. PG is a primary structural component of the bacterial wall that surrounds the cell, maintaining internal turgor and morphology (72). PG synthesis is the target for β-lactam antibiotics. Biochemically, PG is a single macromolecule made of glycan strands cross-linked via peptide side chains. These strands are made by polymerization of *N*-acetylglucosamine (GlcNAc) and *N*-acetyl-muramic acid (MurNAc) and interconnected by peptide side branches (73). Synthesis of the PG is a multistep process (Fig. 2) and occurs in three different locations in the cell. It starts in the cytoplasm, where in the first stage the starter unit of PG biosynthesis, fructose-6-phosphate, is converted into UPD-*N*-acetylglucosamine by a series of reactions catalyzed by **GlmS**, **GlmM**, and GlmU and then into UPD-*N*-acetylmuramate through the activities of **MurA**, MurZ, and **MurB**. In the next steps, performed by **MurCDEF** PG ligases, a pentapeptide side chain (L-Ala-D-Glu-L-Lys-D-Ala-D-Ala) on UDP-MurNAc is synthesized (73). Impaired transport of amino acids, particularly alanine, at this stage leads to increased susceptibility to β-lactams (74).

In the second stage, the UDP-MurNAc-pentapeptide together with a transport lipid bactoprenol form, in an MraY-catalyzed reaction, UDP-P-P-MurNAc-pentapeptide or lipid I. Then, MurG adds UDP-GlcNAc to lipid I, and thus, a final PG building block, UDP-P-P-(GlcNAc)-MurNAc-pentapeptide or lipid II, is synthesized (75). Subsequently, a family of peptidyltransferases (**FemX**, **FemA**, and **FemB**) complete a pentaglycine bridge on the pentapeptide chain, which results in lipid II-Gly_5 (76). For this step, a constant supply of glycine is regulated by **GlyS** (77) and downregulation of ***glyS*** leads to immediate resensitization to β-lactams in MRSA (78). Additionally, the D-Glu in the side chain is amidated to D-glutamine by **GatD** and **MurT** enzymes (79). This is required for optimal polymerization of PG by PBPs. Next, the modified lipid II-Gly_5 is exported to the outer leaflet of the plasma membrane by lipid translocase **MurJ** (80). Many of the genes involved in the intracellular stages of PG synthesis as well as genes that are crucial for divisome formation, ***ftsZ*** and ***ftsA***, have been described as *aux* (Table 2) (54, 78, 81–85).

Translocation of the lipid II-Gly_5 outside the cell serves as a signal for recruitment of **PBP2** and PBP2A (in MRSA), and the third stage of PG biosynthesis begins. During this stage, linear chains of the lipid II-Gly_5 are polymerized into mature PG by several penicillin-binding proteins and other enzymes (86). Each of these proteins plays an important role in this process: **PBP1**, a monofunctional transpeptidase, is required for initiation of PG biosynthesis and separation of the two daughter cells (87); **PBP2**, a bifunctional transpeptidase-transglycosylase, performs transglycosylation of disaccharide units and transpeptidation of the pentapeptide chains during synthesis of the main PG layer; whereas monofunctional transpeptidase **PBP4**, which is nonessential under laboratory conditions, performs additional polymerization of the PG layer (88). In the presence of β-lactam antibiotics, MRSA still requires the presence of the native **PBP1** and **PBP2** (78, 89). **PBP2** remains essential, probably because of its transglycosylase activity, as the heterologous PBP2A can perform only transpeptidase cross-linking (89). In addition, as the catalytic activity of **PBP2** is inhibited, the PG synthesized under such conditions has an unsatisfactory level of cross-linking that makes **PBP4** an essential auxiliary factor for methicillin resistance (90).

The MurNAc residues of the PG can be decorated by a large glycopolymer known as wall teichoic acid (WTA). WTA plays a significant role in growth, morphology, and virulence of *S. aureus,* and the process of WTA biosynthesis has been elucidated (52). The first enzymatic reaction in WTA synthesis is performed by the MraY paralog **TarO**, which forms the initial WTA precursor (Fig. 2). Further extension and decoration of this precursor, its polymerization, glycosylation, export, and D-alanylation are catalyzed by

different **Tar** and **Dlt** proteins, whereas its attachment to PG is mediated by the LCP family of enzymes (91). Screening of *S. aureus* mutants using an antisense-mRNA approach (78) has shown that depletion of Tar proteins has a severe negative effect on β-lactam resistance (Table 2). It has been speculated that in MRSA, WTA serves as an adjunct for PBP2A to perform PG cross-linking (92, 93).

The third important structural polymer of the bacterial cell wall is lipoteichoic acid (LTA). It consists of glycerol phosphate building blocks decorated with D-alanine residues. The biosynthesis of LTA has been studied and characterized (94). The key enzyme in this process is **LtaS**, LTA synthase, which polymerizes the LTA backbone chain at the outside of the cell membrane. Production of LTA is regulated at the posttranslational level by signal peptidase **SpsB**, and loss of it in the MRSA background leads to a decrease in β-lactam resistance, as does an ***ltaS*** mutant, which also leads to severe morphological defects and survival only under osmotically stabilizing conditions with high concentrations of sucrose (92). In fact, **Δ*ltaS*** mutants tend to acquire compensatory mutations in other genes, such as *gdpP*, *sgtB*, *mazE*, *clpX*, or *vraT* (95). In a screen of an ordered library of transposon mutants, **AuxA** and **AuxB** have been identified as being required for both LTA stability and high-level β-lactam resistance (96).

An interesting interaction between metabolism, protein modification, and β-lactam resistance has recently been described (97). Mutations in ***sucC*** or ***sucD*** lead to reduced resistance, concomitant with an increase in the succinylation of multiple proteins. These include the major PG hydrolase Atl, which results in reduced enzyme activity. It is the modulation of cell wall homeostasis enzymes that is proposed to result in the perturbation of resistance (97).

The involvement of Aux factors from across the spectrum of different cell wall components highlights that optimal PG synthesis requires the wider context of overall cell wall homeostasis.

Cell wall stress stimulon (VraSR, TarA, GlmS, FmtA, PBP2, SgtB). The sensor-regulator system **VraSR** is a sensor of cell wall integrity and activates the genes required for cell wall repair in case of inhibition of its synthesis or damage caused by cell wall-targeting antibiotics (98). This system consists of the histidine kinase **VraS**, which in response to cell wall damage signals undergoes autophosphorylation and then activates its cognate response regulator, **VraR**. When activated, **VraR** acts as a transcriptional regulator and modulates transcription of nearly 40 genes (99). The third component, VraT (YvqF), is a putative membrane protein. Its gene is cotranscribed with other components of the system, and VraT is required for correct induction of the **VraSR**-mediated cell damage response, but its role remains unclear (100). The **VraSR**-dependent regulon is known as the cell wall stress stimulon (CWSS) and consists of many genes required for cell wall synthesis and homeostasis and thus includes some auxiliary factors, namely, ***tarA*** (*tagA*), ***glmS***, ***pbp2***, ***sgtB***, and the ***vraSR*** operon itself (99, 101). It has also been suggested that auxiliary factor **FmtA** is controlled by **VraR**, but in this case the modulation of expression is indirect (99, 101, 102). The exact nature of the signal(s) required for autophosphorylation of **VraS** and therefore the triggering of CWSS in the natural environment remains unknown. However, the **VraSR**-mediated response in *S. aureus* can be caused by loss of LCP proteins (103). The **LCP** proteins act as WTA ligases, and their loss results in defective cell separation, increased β-lactam susceptibility, changes in cell wall properties, and other defects (104, 105).

The **VraSR** regulon also contains ***prsA*** and ***htrA1***, which are required for the correct maturation of PBP2A. The chaperone **PrsA** is needed for appropriate folding of PBP2A at physiological temperatures (106), whereas the serine protease **HtrA1** ensures proteolysis of misfolded PBP2A molecules (107). The synergistic role of these proteins for PBP2A quality control is confirmed by the fact that single deletions of ***prsA*** or ***htrA1*** in the model MRSA strain COL had only a mild effect on β-lactam resistance, whereas a double deletion led to a significant drop (107).

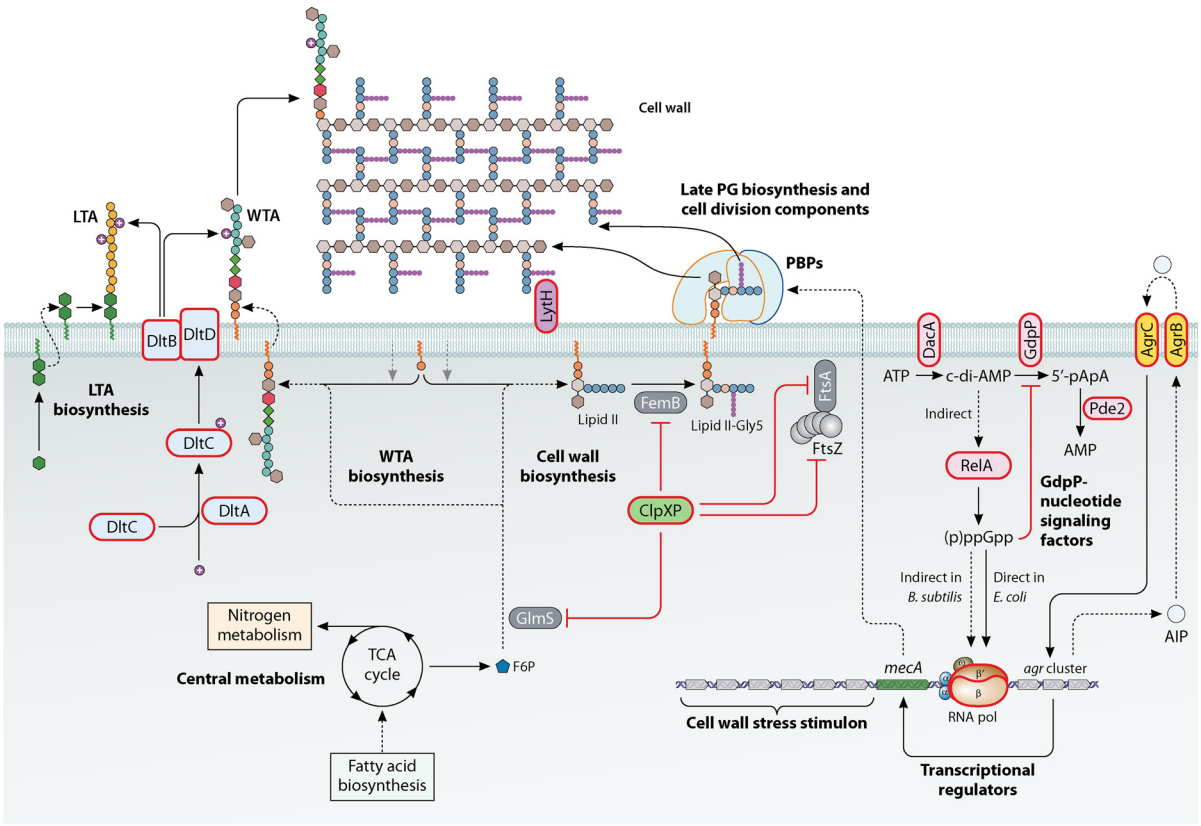

FIG 3 Schematic model of the range of Pot factors involved in β-lactam resistance by MRSA. The Pot factors have a variety of largely pleiotropic functions in cellular physiology and cell wall homeostasis. Potentiators have a red outline; other components have a black outline.

VraSR is not only limited to the regulation of genes involved in cell wall homeostasis but also, by downregulating the *agr* operon, can modulate quorum sensing and virulence of *S. aureus* (108). Natural products that effect **VraSR** have been found to resensitize MRSA to cell wall antibiotics, and thus this regulatory mechanism is a target for the development of new antimicrobial agents (109).

VraSR has likely evolved as a key regulator, monitoring the physiological status of the essential cell wall. To what it responds is currently unknown, and antibiotic resistance can be used as a tool to probe its important cellular functions.

Stress-associated sigma factors (SigB and PrsS). Alternative sigma factors provide one of the many mechanisms for *S. aureus* to respond to its environment, both internal and external. **SigB** is an alternative sigma factor that is involved in a wide variety of cellular processes. This includes control of stress response (110) and biofilm formation (111). Early studies of *S. aureus* transposon mutants and deletion mutants (112, 113) have demonstrated that disruption or deletion of ***sigB*** leads to a decrease of methicillin resistance in strain COL. This could be due to reduced *mecA* expression in the ***sigB*** mutant (114). Another two proteins that interact with **SigB**, global regulator **SarA** and the **PknB** kinase, are important for methicillin resistance (114, 115), but the precise mechanism of this still has to be elucidated.

The extracytoplasmic function (ECF) sigma factor **PrsS** is another regulatory protein required to support methicillin resistance in USA300 (116). This could be due to decreased accumulation of PBP2A and another auxiliary factor, a zinc-dependent endopeptidase, **HmrA** (117).

Potentiator (Pot) Factors: Mutations Increase β-lactam Resistance

Pot factors (Fig. 3; Table 3) provide a window from which to view the impact of PBP2A on the cell and how its role can be most optimally accommodated. They provide

TABLE 3 Potentiator (Pot) factors: mutations increase β-lactam resistance

Gene/operon	SAOUHSC	Functional class	Function	Reference(s)
clpXP	01778/00790	Protein stability	ATP-dependent Clp protease	118
gdpP	00015	Nucleotide signaling	Phosphodiesterase, hydrolyzes cyclic-di-AMP	128
pde2	01812	Nucleotide signaling	Phosphodiesterase, hydrolyzes c-di-AMP and pApA to AMP	129
relA	01742	Nucleotide signaling	Bifunctional synthase and hydrolase of (p)ppGpp alarmone	69
rpoB/rpoC	00524/5	Genetic information processing	DNA-directed RNA polymerase β/β' subunit	50, 141
agr	02261–02265	Quorum sensing	Global regulator of biofilm formation and toxin production	149
lytH	01739	Cell wall homeostasis	PG hydrolase	155
dlt operon	00868–00872	Cell wall homeostasis	Transfer of D-alanine onto teichoic acids	152

something that PBP2A (and SCC*mec*) alone cannot do alone. Their effect is profound, leading to a multifold increase in resistance. However, their mode of action is largely a mystery. Do they act via PBP2A activity or independently? Unlike Aux, the Pot factors are a more eclectic group of components, whose effects span from pleiotropic regulation of gene expression through cell signaling pathways, protein stability, and cell wall homeostasis. The components that have been published as acting as potentiator factors are shown in parentheses in the headings of the sections below and in Table 3.

Protein turnover and the ClpXP system (ClpXP). The proteolytic activity of ClpXP is required in a wide range of cellular processes, including the modulation of β-lactam resistance (99, 118) and production of the major virulence factor Protein A (100, 119). Thus, inhibition of ClpXP proteolytic activity is an attractive target for developing new approaches for the treatment of infections (120). However, loss of ClpXP leads to conversion to homogeneous high-level β-lactam resistance (118), cell wall thickening, an increase in PG cross-linking, and reduced cell size.

The ATP-dependent ClpXP proteolytic complexes are widely distributed among different bacterial species and are responsible for targeted protein degradation during the bacterial life cycle (121). The complex is made of two distinct proteins, an ATPase called ClpX and a peptidase called ClpP. The function of ClpX is to recognize, unfold, and translocate proteins tagged for degradation in the proteolytic chamber of ClpP. Besides this, the ClpX subunit also can act independently as a molecular chaperone that facilitates correct folding of newly synthesized proteins (121). The ClpP subunit, as well, can act on its own, but because of the small size of its proteolytic chamber, ClpP is able to independently degrade only small peptides, or it may also interact with other ATPase subunits with different recognition specificities, such as ClpC or ClpA. Loss of ClpP has a more profound effect on β-lactam resistance than loss of ClpX, while ClpC has no role, suggesting that ClpP modulates β-lactam resistance through ClpX/C-independent mechanisms (118). Studies using an inactive version of ClpXP that traps protein substrates inside its proteolytic chamber (122) shows that some of the *aux* gene products, including ***femB***, ***glmS***, ***ftsZ***, and ***ftsA,*** are direct targets for ClpXP. Thus, elimination of ClpXP proteolytic activity could stabilize auxiliary factors and lead to an increase in resistance. Comparison of transcriptomic data for an *S. aureus clpX* mutant (lacking both ClpX chaperone and ClpXP protease activities) and a mutant expressing a $clpX_{I265E}$ variant of the gene (only the chaperone activity is preserved) demonstrated the modulation of many auxiliary factors that are required for methicillin resistance (123). This includes components of PG biosynthesis (*ftsL* and ***pbp1***) and members of the type VII secretion system. These findings suggest that changes in β-lactam tolerance might still be solely connected with the enzymatic properties of the ClpXP complex; however, in the case of *clpX*, advantages gained through abolishment of the ClpXP activity are simply counterbalanced by the negative impact that absence of the ClpX chaperone activity would have.

Deletion of *clpX* in *S. aureus* can lead to suppression of phenotypes observed as a result of loss of other factors involved in antibiotic resistance. For example, clinical isolates of a daptomycin-resistant *S. aureus* mutant (124) that contain $rpoB_{A477D}$ and exhibit high-level tolerance to oxacillin (>256 μg mL^{-1}) during *in vivo* selection acquired a loss-of-function mutation in *clpX*. The *clpX* mutation in these strains partially compensated the negative

fitness cost caused by $rpoB_{A477D}$, but it did not affect the strain's β-lactam or daptomycin tolerance (125). In another study, loss of ClpX activity rendered the auxiliary gene ***ltaS*** (LTA synthase), ordinarily essential under standard laboratory conditions, nonessential (126). The *clpX* mutation compensated for the septum placement defect presented by LTA-negative mutants of *S. aureus*.

Nucleotide-signaling pathways (GdpP, Pde2, RelA). Nucleotide-signaling molecules are indispensable components as they regulate cellular pathways in all forms of life. The role of nucleotide-signaling molecules in methicillin resistance has been examined in a number of studies (50, 69, 127–129). The recent discovery of *S. aureus* c-di-AMP (128), a signaling nucleotide, has highlighted its importance in the homeostasis of cellular nucleotide concentrations during environmental changes (130). c-di-AMP is synthesized by diadenylyl cyclase (DacA) and hydrolyzed by c-di-AMP phosphodiesterase (GdpP) (127, 128). GdpP contains two N-terminal transmembrane domains, a PAS sensory domain, a GGDEF domain, a DHH domain, and a DHH/DHHA1 domain (128, 131). The C-terminal DHH and DHH/DHHA1 domains possess phosphodiesterase activity that hydrolyzes c-di-AMP to pApA (phosphadenylyl-adenosine) and then to AMP (130–132). Several studies have reported that the increased intracellular levels of c-di-AMP due to the disruption of *gdpP* lead to elevated resistance to cell wall-targeting antibiotics (50, 131, 133–136). Increased c-di-AMP levels have been shown to be associated with increased PG cross-linking, indicating the role of c-di-AMP in regulating cell wall characteristics and concomitant resistance to β-lactam antibiotics (128). Also, cells with depleted intracellular c-di-AMP levels are significantly more sensitive to oxacillin and larger in size (127, 137). This suggests a potential function for c-di-AMP in regulating components of the cell wall homeostasis machinery that control the strength of the cell wall. Another interesting observation is that the regulation of membrane potential also influences the level of resistance to β-lactam antibiotics in a *gdpP* mutant. Mutations in *dacA* or *gdpP* lead, respectively, to reduced or increased c-di-AMP, concomitant with reduced or increased membrane potential (137).

In addition to membrane-bound phosphodiesterase (PDE) *gdpP*, a cytoplasmic PDE encoded by *pde2* which preferentially hydrolyzes pApA to AMP, ensuring tight control of cellular c-di-AMP levels, was recently characterized (129). Interestingly, *pde2* mutation leads to increased resistance to oxacillin similar to that of *gdpP* mutation in strains of different backgrounds (50, 129). Collectively, the transition from low-level to high-level methicillin resistance is associated with c-di-AMP levels potentially by regulating cell wall synthesis and allowing cells to cope with membrane and cell wall damage upon exposure to β-lactam antibiotics.

Mwangi et al. (69) identified point mutations in the *relA* gene, encoding a (p)ppGpp synthetase which triggers the stringent response, to be a positive genetic determinant for methicillin resistance. The turnover of (p)ppGpp is tightly controlled by *relA* (134, 138); however, upon nutrient starvation, which causes the stringent response, cells accumulate high levels of (p)ppGpp in the bacterial cell which slow down the translation of gene products involved in macromolecular biosynthesis (139). Interestingly, Corrigan et al. (64) demonstrated that cells with high levels of (p)ppGpp inhibited the hydrolysis of intracellular c-di-AMP by GdpP, resulting in increased levels of cellular c-di-AMP. Moreover, high-levels of c-di-AMP were shown to activate the production of (p)ppGpp via an unknown mechanism, linking the two different nucleotide-signaling pathways (64). This, in combination with increased PG cross-linking associated with high-levels of intracellular c-di-AMP and upregulation of ***pbp4*** in a *gdpP* mutant (64), suggests that these pathways prepare the cell to withstand antibiotic intervention, but mechanistic insights are elusive.

RNA polymerase (RpoB, RpoC). Mutations in the genes *rpoB* and *rpoC*, which encode the two largest subunits of RNA polymerase, β and β', respectively, are well known factors that can change levels of antibiotic resistance (140). In terms of methicillin resistance, however, the importance of *rpoB*/*C* mutations has quite often been overlooked, as these mutations are identified along with mutations in other potentiators, such as *relA* (66). Now that whole-genome sequencing is a routine procedure, the

significance of *rpo* mutations in conversion from heterogeneous to homogeneous methicillin resistance is highlighted (50).

Those *rpo* mutants with an increased resistance to β-lactams have a pleiotropic phenotype (50, 125, 141). This includes a prolonged doubling time and increased cell wall thickness. In addition, *S. aureus* cells harboring $rpoB_{A477D}$ are smaller in size (125). In relation to changes in gene expression, β-lactam-associated mutations in the genes encoding RNA polymerase lead to increased expression of *mecA* (50), which also correlates with increased concentration of PBP2A (141). The increased resistance to methicillin cannot, however, be attributed to PBP2A levels, as overexpression of *mecA* from a multicopy plasmid did not result in high-level resistance (50). The introduction of *mecA* into *S. aureus* and subsequent acquisition of the *rpo* mutations leading to high-level resistance have been established (50). The introduction of *mecA* alone, in the MSSA SH1000 strain background, leads to increased expression of the genes involved in metabolism of pyruvate (*ldh1*, *lctP*, *ald1*, *adhE*, *adh*, *pflA*, and *pflB*) and nitrogen (*nirR*, *nirB*, *nirD*, *nasF*, *narG*, *narH*, *narT*, and *narJ*), as well as genes of oxygen-independent ribonucleotide reductase (*nrdD* and *nrdG*). This set of genes is usually associated with anaerobic growth. After the *mecA*-containing strains acquired *rpo* mutations, the expression level of these genes returned to the parental level. This suggests that respiration is impaired by *mecA*, and this is suppressed by an *rpo* mutation, as was verified by oxygen utilization experiments (50).

Biochemical characterization of RNA polymerase with mutated β or β' subunits associated with high-level methicillin resistance (50) revealed changes in transcription initiation elongation and RNA polymerase, with a mutated β subunit having lower affinity for SigA. These changes could result in differential gene expression compared to that of the parental strain. It is important to point out an interaction loop that exists between stress response regulator Spx and two potentiators, RNA polymerase and the ClpXP proteolytic complex. Spx controls expression of its regulon by interacting directly with α subunit of RNA polymerase. Spx is essential, but its loss can be compensated by a mutation in *rpoB*, associated with rifampin resistance (142). The $rpoB_{A477D}$ mutation (125) led to an increased concentration of Spx. Finally, the level of Spx in the cell is regulated by ClpXP proteolysis (123). Thus, both potentiators, *rpo* and *clpXP*, increase resistance to β-lactams with a concomitant increase in Spx levels.

Quorum sensing and the accessory gene regulator system (Agr). The accessory gene regulator cluster (*agr*) is a global regulatory system that governs quorum sensing and virulence in *S. aureus*. Inactivation of *agr* leads to a significant increase in the rate of conversion from heterogeneous to homogeneous resistance, whereas complementation of the *agr* mutant reverses this effect (143, 144).

The products of the *agr* cluster (AgrA to -D) produce and respond to the buildup of autoinducing peptide (AIP) in the environment as a quorum sensing system. The Agr system modulates the production of a range of other regulators, for example, **SarA**, SarR, SrrAB, SarX, CodY, and **SigB**, in a direct or indirect manner (145). Another gene in the *agr* cluster encodes the RNAIII transcript, which is a multifunctional RNA that can act as antisense controlling posttranscriptional regulation of many components (146). Transcriptomic studies (147) demonstrated that RNAIII modulates expression of major virulence factors and transcriptional regulators.

Health care-associated MRSA (HA-MRSA) strains have a correlation between high expression levels of *mecA* and a repressed or dysfunctional *agr* cluster, with concomitant reduced toxin production and lower virulence, whereas community-associated MRSA (CA-MRSA) strains usually have lower PBP2A but increased *agr* expression (136, 148). The rationale for this trade-off may relate to the changes in PG structure caused by PBP2A, thus reducing the ability of the Agr system to respond to increased AIP concentrations and to effectively autoactivate the *agr* cluster (148). Recent experiments with USA300, a CA-MRSA strain that has low-level *mecA* expression, showed that inactivation of the *agr* cluster led to changes in *mecA* expression (149), suggesting a cross-regulation between *agr* and *mecA*. Inactivation of the *agr* cluster also leads to decreased susceptibility to oxacillin and ampicillin. The *agr* mutation results in increased concentrations of long chain

fatty acids in the cytoplasmic membrane, possibly contributing to increased membrane stability and thus affecting β-lactam resistance (149, 150).

Cell wall homeostasis (Dlt, LytH). As described above, many components involved in PG biosynthesis are auxiliary factors required for high-level β-lactam resistance (Table 3). However, inactivation of genes within the *dltABCDX* operon (151) in MRSA strain KAN96 has the opposite effect (152). Products of this operon catalyze decoration of LTA and WTA by D-alanine residues and suggests that the resulting changes in cell wall structure may reduce its susceptibility to β-lactam antibiotics (152).

Cell wall homeostasis is governed by PG synthesis and hydrolysis to permit growth and division. The PG hydrolase LytH is an amidase that targets uncross-linked PG (153). In early studies, laboratory mutants as well as clinical isolates lacking LytH activity demonstrated increased methicillin resistance (154, 155), although this has been questioned (153).

CONCLUDING REMARKS

High-level β-lactam resistance requires the acquisition of a gene encoding an exogenous PBP, generally *mecA*. However, the presence of PBP2A brings with it several cellular challenges. This protein has to be able to take over the essential transpeptidase activity of endogenous PBPs in the presence of β-lactams but in doing so must not significantly interfere with PG synthesis in the absence of antibiotics. This creates a delicate balancing act for the cell, requiring the correct functioning of a number of components (Aux factors) to support resistance. These have roles in many processes that permit optimal PG synthesis and the flow of precursors. However, there is also an intriguing set of genes in which mutations promote the development of high-level resistance (Pot). Recent transcriptional analysis has provided a glimpse into how the potentiator *rpo* may facilitate the ability of PBP2A to function optimally, without disrupting important physiological processes (50). It is interesting to speculate as to whether the Pot factors all act independently or are part of a continuum that facilitates high-level resistance. Elucidating their individual and collective roles in MRSA and MSSA will reveal insights into fundamental physiology and how this underpins the development of clinically relevant antibiotic resistance.

The clinical importance of MRSA reveals that whatever molecular mechanisms are at play, they do not unduly affect the fitness of resistant strains that are able to proliferate and cause disease. This is important, as it reveals that harboring SCC*mec* and the *pot* mutation in *rpo* do not in themselves disadvantage MRSA in the hospital environment, as shown by the epidemic spread of strains around the world. The acquisition of *mecA* and *rpo* results in high-level MRSA with concomitant slower growth *in vitro* but no change in the ability of the strain to cause disease in a mouse sepsis model (50). However, this is nuanced by the SCC*mec* type, where CA-MRSA with the smaller type IV element is able to maintain growth rate and toxin production levels *in vitro*, compared to HA-MRSA with the larger type II, suggesting a role in the ability of strains to compete in the community setting (156). CA-MRSA has evolved several times, where an evolutionary compromise has been achieved between maintaining antibiotic resistance and enhanced pathogenic capability but without sacrificing overall fitness (157).

The complex set of genetic determinants that support β-lactam resistance sets up a number of questions to be addressed in order to explain how high-level resistance is maintained. These questions are not only interesting from an antibiotic resistance perspective—it must be remembered that resistance emerges from the basic mechanisms of bacterial physiology that underpin growth and division.

UNANSWERED QUESTIONS

What is the role of the SCC*mec* type and the chromosomal background in the complement of identified Aux and Pot factors? To date, studies have taken place over a large range of strains, which has made underlying principles difficult to elucidate.

What are the individual and collective roles of the Aux and Pot factors in resistance? While there are clear themes among the Aux factors, how they and the Pot factors work to support high-level resistance is largely obscure.

What is the effect of PBP2A on *S. aureus* in the absence of antibiotics? PBP2A is an exogenous enzyme that has evolved outside of *S. aureus*, and its presence stresses the cell through mechanisms unknown.

How is PBP2A able to substitute for the endogenous enzymes in PG synthesis in the presence of antibiotics? In the presence of antibiotics, PBP2A takes on the herculean task of all of the endogenous transpeptidases to allow growth and division.

Can an understanding of Aux and Pot factors be exploited to combat the scourge of MRSA? Approaches have already been developed to reduce high-level resistance, and it is these, and others, that may prove clinically important in the future.

What can we learn about the fundamental principles of growth and division from elucidation of MRSA resistance mechanisms? Being MRSA is not essential for the cell and provides a tractable experimental system in which to dissect those physiological processes required for life.

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We declare there are no conflicts of interest.

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