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Diversity of peptidoglycan structure—Modifications and their physiological role in resistance in antibiotic producers

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ABSTRACT

Peptidoglycan (PG) is a bacteria specific cell surface layer that ensures the bacterial shape and integrity. The two actinomycetes *Amycolatopsis balhimycina* and *Microbispora* sp. PTA-5024 are producers of PG targeting antibiotics. To prevent the binding of their secreted product to their own PG, they developed specific self-resistance mechanisms. Modifications of PG, which are applied by both strains, are the introduction of amide-residues at the PG precursors and the alternative crosslinks within the nascent PG.

The PG modifications found in *Microbispora* sp. PTA-5024 seemed to be an intrinsic characteristic of the genus *Microbispora*, rather than a specific mechanism of NAI-107 resistance. In contrast, the modifications in *A. balhimycina* represent an alternative way to avoid suicide specific for glycopeptide producers. The different PG modifications reflect the fact that antibiotic producing organisms contain not only one but multiple mechanisms to ensure protection against biologically active molecules produced by themselves.

1. Introduction

A major component of the bacterial cell wall is peptidoglycan (PG). It is a heteropolymeric layer being located on the exterior of the cytoplasmic membrane, completely enclosing the bacterial cell and providing the bacterial shape and integrity. As PG is an indispensable feature for viability in almost all bacteria as well as it is not present in human cells, intermediates and enzymes of its synthesis are optimal targets for antibacterial agents. Examples for extensively studied classes of PG-targeting antibiotics are the β-lactams and glycopeptide antibiotics (GPAs) in clinical use as well as the lanthipeptides in preclinical stage of development (Ongey et al., 2017). Most of those antibiotics originated from the phylum *Actinobacteria* (Barka et al., 2016). Since the producers must protect themselves from the attack of antibiotics to avoid suicide, they developed self-resistance mechanisms. In order to understand the development of antibiotic resistance in pathogens, it is important to consider those reservoirs of resistance genes, which are believed to be horizontally transferred to the clinical pathogenic bacteria.

β-Lactam antibiotic resistance is caused mainly by two mechanisms: antibiotic-degrading enzymes, (β-lactamases) and modification of target sites, (altered penicillin-binding proteins; PBPs). While the major resistance mechanism in pathogenic bacteria is the hydrolysis of the β-lactam ring by β-lactamases, thus inactivating the antibiotics, that in *Streptomyces* is supposed to be the provision of PBPs possessing low affinities to β-lactam antibiotics (Ogawara, 2016).

In this review we will focus on the resistance mechanisms of two actinomycetes producing antibiotics targeting PG synthesis; *Amycolatopsis balhimycina,* the producer of the vancomycin-type GPA balhimycin, and *Microbispora* sp. PTA-5024, the producer of the lanthipeptide NAI-107, currently in preclinical development (Jabés et al., 2011; Maffioli et al., 2016). NAI-107 is also known as microsporicin A1 produced by *Microbispora* sp. 107891 (Castiglione et al., 2008; Foulston and Bibb, 2010).

GPAs inhibit cell wall biosynthesis in Gram-positive bacteria by binding the D-alanyl-D-alanine (D-Ala-D-Ala) terminus of PG precursors on the outside surface of the cytoplasmic membrane (Reynolds, 1989) (Fig. 1). Vancomycin remains a drug of choice for treatment of severe methicillin resistant *Staphylococcus aureus* (MRSA) infections. In MRSA as well as in enterococci the GPA resistance mechanism is based on the incorporation of alternative amino acids into the peptide stem, resulting in decreased binding affinity of the GPAs to their target. This target modification is mediated by the VanHAX enzymes, which are encoded on transposon Tn*1546*, originally a part of a vancomycin-resistant enterococci (VRE) conjugative plasmid (Arthur et al., 1993). Vancomycin resistance in *S. aureus* is maintained by retaining an original

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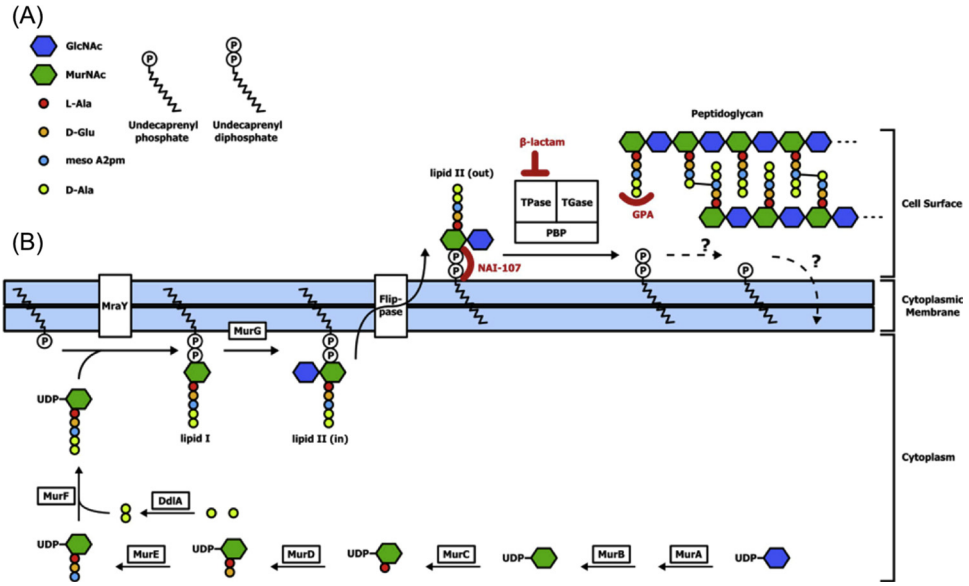

Fig. 1. Peptidoglycan composition and synthesis. A) The composition of a peptidoglycan (PG) precursor is illustrated. B) Scheme of PG synthesis is shown. Abbreviations: GlcNAc(*N*-acetylglucosamine) in blue, MurNAc (*N*-acetylmuramic acid) in green, L-Ala (L-alanine) in red, D-Glu(D-glumatic acid) in orange, *m*Dap (*meso*-diaminopimelic acid) in light blue, D-Ala (D-alanine) in purple, GT(glycosyltransfer),TP (transpeptidation). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

enterococcal plasmid or by a transposition of Tn*1546* from the VRE plasmid into a staphylococcal resistent plasmid (Périchon and Courvalin, 2009; Zhu et al., 2010). To better understand the molecular mechanism by which the VanHAX confers resistance (McGuinness et al., 2017), it is necessary to understand primary the self resistance mechanism in GPA producers, exemplarily demonstrated in this review by *A. balhimycina* (Paragraph 3.1).

Lanthipeptides like nisin and NAI-107 exhibit antimicrobial activity by binding to the pyrophosphate moiety of lipid II (Brötz et al., 1998; Münch et al., 2014) a membrane-bound advanced intermediate involved in the biosynthesis of the cell wall (Fig. 1). By doing so, they inhibit the transglycosylation step in cell wall biogenesis and sequesters lipid II from its functional location (Breukink et al., 1999; Münch et al., 2014). In contrast to NAI-107, the nisin-lipid II complex leads to formation of pores in the membrane causing cell death (Wiedemann et al., 2001; Garg et al., 2014;). Known resistance determinants against lanthipeptides are ABC-transporters (as shown for epidermin) (Otto et al., 1998) as well as lipoproteins (as shown for nisin and subtilin) (Stein et al., 2003, 2005) being located within the PG network. In *Microbispora* self-resistance against NAI-107 is mediated by the lipoprotein MlbQ and the ABC-transporter MlbYZ together with the transmembrane protein MlbJ, which might act as a substrate-binding protein. MlbQ posseses an unstructured N-terminal region and a small, globular C-terminal domain. The hydrophobic surface patch of the protein, is proposed to bind to NAI-107 (Pozzi et al., 2016). It is supposed that these determinants repel the antibiotic in order to prevent the binding of NAI-107 to its target.

Additional analyses of the PG precursors and the mature PG revealed that both producers, *A. balhimycina* and *Microbispora* sp., exhibit modifications on the peptide stem precursors as well as on the PG network occurring during polymerization.

Modifications of GlcNAc or MurNAc in the glycan backbone like *N*-deacetylation, *N*-glycolylation or *O*-acetylation may also play a role in resistance against lysozyme treatment (Raymond et al., 2005), pathogenicity (Bera et al., 2006) and virulence (Aubry et al., 2011). Recent studies by Chang et al. (2017) showed that *O*-acetylation also occurs in vancomycin-resistant *Enterococcus faecalis* (VRE) spp. especially when they are exposed to vancomycin, predominantly in regions where reduced crosslinking appeared. They conclude that these modifications protect against carboxypeptidases and autolysins.

However, so far, no modifications of the carbon chain have been observed in *A. balhimycina* and *Microbispora*. In this mini review we will focus on those target modifications, which affect the mode of action of antibiotics used against Gram-positive bacteria. We will evaluate their contribution to protection against GPAs and lanthipeptides and their relevance in non-antibiotic producers and in the antibiotic producers *A. balhimycina* and *Microbispora* ATCC PTA-5024.

2. Synthesis of the peptidoglycan

In this paragraph we provide a brief overview of the PG synthesis in general. These fundamentals build the basis for the explanation of the modifications, which are introduced by Gram-positive bacteria in order to avert the attacks of β-lactams, GPAs and lanthipeptides (Paragraph 3 and 4).

The PG synthesis occurs in three distinctive compartments of the bacterial cell, namely the cytoplasm, the cytoplasmic membrane, and the cell surface (Liu and Breukink, 2016) (Fig. 1): (1) In the cytoplasm the lipid II synthesis takes place; lipid II consists of the sugar moieties on which the peptide stem is attached (undecaprenyl-pyrophosphoryl-MurNAc-(pentapeptide)-GlcNAc). (2) The lipid-anchored PG precursor is translocated to the extracellular face of the cytoplasmic membrane. (3) The polymerizing of the nascent PG occurs at the cell surface.

2.1. Synthesis of the peptide stem in the cytoplasm

The PG synthesis starts in the cytoplasm, where the activated precursors uridine diphosphate (UDP)-GlcNAc and UDP-MurNAc are produced. UDP-GlcNAc is synthesized from the glycolytic intermediate D-fructose-6-phosphate (fructose-6 P) in successive reactions catalyzed by three enzymes (GlmS, GlmM and GlmU) (Rodríguez-Díaz et al., 2012). MurA transfers enolpyruvate from phosphoenolpyruvate to UDP-GlcNAc (Lovering et al., 2012; van Heijenoort, 2001). The resulting product, UDP-GlcNAc-enolpyruvate then undergoes a MurB catalyzed reduction forming UDP-MurNAc. Following the production of UDP-MurNAc in the cytoplasm, a series of ATP-dependent amino acid ligases (MurC-F) proceed the stepwise addition of the pentapeptide side chain to UDP-MurNAc: MurC is responsible for the first addition of L-Ala to UDP-MurNAc, MurD for the addition of D-Glu to the MurC product UDP-MurNAc-L-Ala. MurE catalyzes the addition of the third peptidyl residue, L-Lys or mDap, to form UDP-*N*-acetylmuramyl-tripeptide. MurF catalyzes the next cytoplasmic step in the PG synthesis with the addition of D-Ala-D-Ala to the UDP-MurNAc-L-Ala-D-Glu-mDap acid product of MurE (Lovering et al., 2012). The dipeptide D-Ala-D-Ala is formed by the ligase DdlA.

2.2. Membrane associated steps of the peptidoglycan synthesis

The first membrane-associated step in the PG synthesis is catalyzed by the integral membrane protein MraY. This reaction involves the transfer of the MurNAc-pentapeptide from the cytoplasm to an undecaprenyl phosphate carrier (C_{55}-P) on the cytoplasmic side of the membrane (Lovering et al., 2012). The result is the formation of uridine monophosphate (UMP) and the undecaprenyl-pyrophosphoryl-MurNAc-pentapeptide, commonly referred to as lipid I (Liu and Breukink, 2016). The glycosyltransferase MurG transfers a GlcNAc moiety from UDP-GlcNAc to lipid I in order to produce undecaprenyl-pyrophosphoryl-MurNAc-(pentapeptide)-GlcNAc, commonly referred to as lipid II.

The translocation across the cytoplasmic membrane has recently been enlightened: In the Gram-negative model organism *E. coli* MurJ flips lipid II across the cytoplasmic membrane (Sham et al., 2014; Zheng et al., 2018), whereas in the Gram-positive model organism *Bacillus subtilis* besides MurJ a second flippase, Amj, was shown to translocate lipid II (Meeske et al., 2015).

2.3. Polymerization of the peptidoglycan

After flipping to the outer leaflet of the cytoplasmic membrane the incorporation of lipid II in the polymerizing PG occurs and releases undecaprenyl-pyrophosphate.

The strands are polymerized by membrane-embedded PG glycosyltransferase (PGT) enzymes using lipid II. The polymerized glycans are then crosslinked via the formation of amide bonds between attached peptides by penicillin binding proteins (PBP). They hydrolyze the terminal D-Ala of the pentapeptide stem of lipid II and form a 3-4 crosslinkage between neighbouring stem peptides (Cho et al., 2014). PBP possess a modular structure and are classified according to their activity. Class A PBPs are bifunctional membrane enchored enzymes with both PGT and transpeptidase (TP) activity, whereas class B PBPs are monofunctional TP. Most of them also have non-catalytic domains of which some are involved in interactions with other proteins to regulate the enzymatic activities. Class C PBPs are PG hydrolases with, D, D-carboxypeptidase or endopeptidase activity (EPases). EPases cleave the peptide cross-links, and D-ala carboxypeptidase (CPases) hydrolyse the terminal amino acid residue of the peptides. The action of the PBPs ultimately results in the PG layer that is responsible for the shape and rigidity of the bacterial cell (Egan et al., 2015).

3. Effects of the lipid II peptide stem modifications

Since the PG is a favoured target of antibacterial agents bacteria developed strategies to evade these attacks by introducing different modifications. In the following paragraphs we will focus on those PG modifications, which are known to have an effect on the mode of action of antibiotics targeting PG synthesis in Gram-positive bacteria. We will point out the modifications, which play a role in the producers *A. balhimycina* and *Microbispora* sp. in order to prevent the binding of antibiotics to their target (Fig. 2).

3.1. Incorporation of alternative amino acids into the peptide stem

The most prominent modification of the peptide stem is the replacement of D-Ala-D-Ala by Ala-D-Lac or by D-Ala-D-serine (Ser), which leads to resistance to GPAs. GPA resistance was first described in pathogenic enterococci. In these bacteria the operon *vanRSHAXY* is responsible for reprogramming of the PG precursor biosynthesis. The exchange of the fifth amino acid from D-Ala to D-Lac or D-Ser decreases substrate-binding affinity (1000-fold) between the di(depsi)peptide and the lipid II-targeting GPA (Leclercq et al., 1988; Bugg et al., 1991) like vancomycin, teicoplanin or balhimycin. This mechanism is the most complex resistant mechanism known so far. It is presumed, that the corresponding resistance genes are not evolved in pathogenic bacteria but co-evolved in the producers with the capability to synthesise GPAs. Subsequently they were transferred to the pathogens using horizontal gene transfer mechanisms potentially involving plasmids, integrons, or transposons (Jiang et al., 2017; Peterson and Kaur, 2018; Vogelmann et al., 2011). The *vanHAX* genes provide a striking example of a strong connection between antibiotic resistance genes in clinical isolates and those found in antibiotic producing bacteria, since there is a considerable protein sequence similarity as well as a conserved arrangement and organization of genes (Barna and Williams, 1984; Marshall et al., 1998; Peterson and Kaur, 2018).

VanH, a D-stereospecific lactate dehydrogenase, converts pyruvate to D-Lac (Arthur et al., 1991). VanA ligates D-Ala and D-Lac to D-Ala-D-Lac-depsipeptide (Bugg et al., 1991). VanX is a specific carboxypeptidase, which eliminates the remaining D-Ala-D-Ala-dipeptides avoiding their incorporation into the cell wall precursors (Wu et al., 1995).

Nine different glycopeptide resistance phenotypes have been described in vancomycin resistant enterococci. The activation of the VanA, B, D, F, and M types resulted in the formation of precursors ending on D-Ala-D-Lac. The VanC, E, G, and N types do not exchange the terminal D-Ala with D-Lac but rather with a D-Ser using a different set of enzymes (Courvalin, 2006).

Close homologues of *vanH, vanA,* and *vanX* are present in the genome of *A. balhimycina* and other GPA producing actinomycetes (e.g. Beltrametti et al., 2007; Frasch et al., 2015; Kilian et al., 2016; Marshall and Wright, 1997, 1998; Schäberle et al., 2011; Sosio et al., 2004; Spohn et al., 2014; Thaker et al., 2013). In contrast to the pathogenic GPA resistant bacteria, where the expression of the *vanHAX* genes is tightly regulated by the two-component system VanRS, GPA producers express the *vanHAX* genes constitutively. In *A. balhimycina* the *vanHAX* genes are located more than 2 Mb apart from the genes encoding the two component system VnlRS, which are located in the balhimycin biosynthetic gene cluster (Frasch et al., 2015; Kilian et al., 2016). A constitutive expression of the *vanHAX* genes has also been observed in *Amycolatopsis japonicum*. *A. japonicum* contains a biosynthetic gene cluster, which encodes the synthesis of the GPA ristomycin; this strain is GPA resistant, even under conditions where no GPA is produced (Schäberle et al., 2011; Spohn et al., 2014). A similar observation was made for *Actinoplanes teichomyceticus* the producer of the GPA teicoplanin, which is in medical use. This strain is intrinsically resistant to the GPA (Beltrametti et al., 2007; Marcone et al., 2014; Binda et al., 2018). Its genome of *A. teichomyceticus* harbours the canonical *vanHAX* gene cluster including the *vanRS* two component-regulatory genes associated with the teicoplanin biosynthetic gene cluster (Beltrametti et al., 2007). These examples suggest that GPA producers constitutively express the *vanHAX* genes, modify their peptide stem and thus have an advantage over their competitors. In contrast, non-producers regulate the expression of the resistance genes in response to the presence of the antibiotics.

A peculiarity of *A. balhimycina* resistance is, that even in the absence of the *vanHAX* genes, the strain is able to synthesize PG precursors carrying a D-Lac at the 5th position. It could be shown that an alternative D-Ala-D-Lac ligase takes over the function of VanA. The substrate D-Lac is probably subtracted from the basal metabolic pathways of the strain (Frasch et al., 2015).

3.2. Modifications of the amino acids of the peptide stem

Another modification within the PG network that leads to altered susceptibility to antibiotics is the amidation of the amino acids at position 2 (D-Glu) and 3 (mDap) of the peptide stems. These reactions occur before translocation of the PG precursors across the cytoplasmatic membrane (Münch et al., 2012; Nöldeke et al., 2018; Leisico et al., 2018).

The amidation plays an important role in the pathogenic bacterium

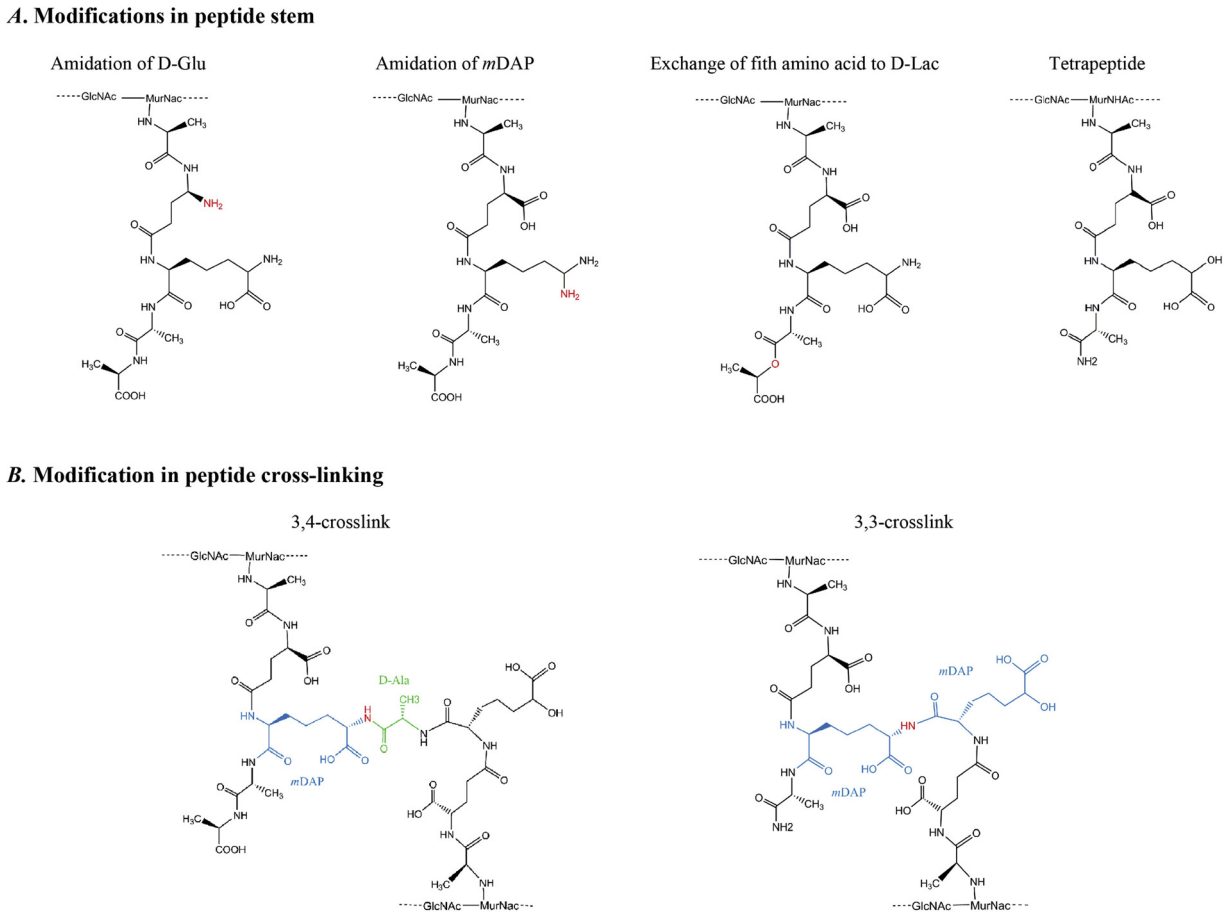

Fig. 2. Modifications of peptidoglycan conferring resistance against PG targeting antibioitcs. (A) Modifications in peptide stem. Alterations are marked in red. (B) Modification in peptide cross-linking amino acids involved in cross-linked are marked in colours as described in Fig. 1. Peptide bond is marked in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

S. aureus. Here the D-Glu is almost completely modified to D-Gln, a reaction mediated by the MurT-GatD-complex. *S. aureus* mutants with a reduced degree of amidation are less viable and show increased susceptibility to methicillin, indicating that targeting the amidation reaction could be a useful strategy to combat this pathogen (Nöldeke et al., 2018).

In *Lactococcus plantarum* (Bernard et al., 2011) the amidation of mDap is catalyzed by the amidotransferase AsnB1. This modification is crucial for the cross-linking of the precursors, the growth and the control of septation (Bernard et al., 2011). Dajkovic et al. (2017) showed that in *B. subtilis* the absence of amidated mDAP causes a lethal deregulation of PG hydrolysis that could only be inhibited by increased levels of Mg^{2+} and suggested that amidation and Mg^{2+} regulate the balance between PG synthesis and hydrolysis.

Additionally, the deletion of the amidotransferase gene in *Corynebacterium glutamicum*, encoding the mDAP amidation, resulted in enhanced β-lactam and lysozyme susceptibility in this mutant (Levefaudes et al., 2015).

In contrast, although detailed structural analysis of the NAI-107 producer *Microbispora* sp. mature PG revealed the amidation of iso-Glu at amino acid position 2 of the peptide stem (Pozzi et al., 2016) *in vitro* studies showed no effects of amidated Glu on the binding affinity of NAI-107 to lipid II. Thus, the α-carboxylic group of Glu is not crucial for NAI-107 binding (Münch et al, 2014).

Furthermore, a substitution of the Ala with Gly or Ser at position 4 or alternatively at position 5 of monomeric tetra- and pentapeptides has been detected. However, no UDP-linked PG precursors in the cytoplasm containing Gly or Ser were found. The presence of Gly and Ser in the monomer muropeptides seems to be the result of a modification, which occurs at the late stage of PG maturation (Pozzi et al., 2016). The exchange of D-Ala in the fourth position for D-amino acids (like Gly) is known to appear as a side reaction of L,D-transpeptidases (Ldts) (Paragraph 4) (Mainardi et al., 2000; Magnet et al., 2008). In the genome of *Microbispora* sp. putative Ldts are encoded, indicating the potential of *Microbispora* sp. to substitute D-Ala for Gly or D-Ser in monomer muropeptides.

PG of *A. balhimycina* is amidated either at mDap or D-Glu as well as at both amino acids. The amidation in *A. balhimycina* does not have a direct impact on GPA resistance. However, it has been shown that amidation may be crucial for the metallo-D,D-carboxypeptidase in lactobacilli (Bernard et al., 2011; Figueiredo et al., 2012; Münch et al., 2012). This carboxypeptidase hydrolyses the C-terminal D-Ala residue of the cytoplasmic PG precursor UDP-MurNAc-pentapeptide. The resulting tetrapeptide acts as a substrate for Ldts (Paragraph 4). In *Enterococcus faecium* the activity of the carboxypeptidase $DdcY_{fm}$ and thereby the formation of the tetrapeptide is controlled by a two-component regulatory system ($DdcRS_{fm}$) (Cremniter et al., 2006; Sacco et al., 2010, 2014). The *ddcRSY*$_{fm}$ genes display striking similarities with the *vlnRS* and *vanY* genes identified in *A. balhimycina*. The fact, that these genes are located in the balhimycin biosynthetic gene cluster indicates a co-evolution of antibiotic production together with protection and emphasise the role of VlnRS and VanY in GPA resistance. Transcriptional analyses of those genes revealed that they are overexpressed in the *A. balhimycina* mutant where the *vanHAX* genes are deleted (Frasch et al., 2015). Furthermore, it has been shown that *vanY* is strictly regulated by VlnRS since in the *A. balhimycina* Δ*vlnRS* mutant

vanY transcription was abolished (Kilian et al., 2016). The involvement of the D,D-carboxypeptidase VanY in GPA resistance have also been demonstrated for *Nonomureaea* sp. producing the teicoplanin like GPA A40926, which is the precursor of the semisynthetic antibiotic dalbavancin, currently in phase III clinical trials (Marcone et al., 2010).

4. Alternative peptidoglycan cross-linking

The final steps of PG synthesis involve polymerization of the glycan strands by PGT and cross-linking of the peptide stems by D,D-TP. They catalyze formation of a peptide bond between the α-carboxyl of D-Ala at the fourth position of a donor stem and the amino group of mDap at the third position of an acceptor stem generating a D-Ala$_4$-mDap$_3$ (3-4) cross-link. The binding of GPAs and lanthipeptides to the precursor prevent the transpeptidation and transglycosylation reactions. On the other hand, the D,D-TP are the target of β-lactams (Goffin and Ghuysen, 1998). One way to circumvent β-lactam resistance is the use of an alternative TP, the Ldt. Ldts are responsible for the cross-links via a peptide bridge, which is formed between the amino acids at position 3 of each stem peptide (3-3 cross-links) resulting in GPA resistant PG.

The Ldt from *E. faecium*, Ldt$_{fm}$, the first functionally characterized TP, cleaves the L-Lys3-D-Ala4 peptide bond of tetrapeptide stems that act as acyl donors in the cross-linking reaction in order to form the 3-3 cross-link between a tetra and a tripeptide (Fig. 1). Ldt$_{fm}$ is devoid of any activity for pentapeptides. Their contribution to PG cross-linking appears to be controlled by the availability of tetrapeptides, provided by the metallo-D,D-carboxypeptidase DdcY$_{fm}$ (Cremniter et al., 2006; Sacco et al., 2010, 2014) (Paragraph 3). In *Mycobacterium tuberculosis* and *Enterococcus faecalis* the Ldt needs amidation of lipid II, especially of mDAP, to catalyze 3-3 cross-linking (Ngadjeua et al., 2018).

In both producer strains, *Microbispora* sp. and *A. balhimycina* analyses of the mature PG demonstrated the presence of dimers with 3-4 cross-links as well as dimers with 3-3 cross-links, suggesting the action of both D,D- and L,D-transpeptidases in cross-linking and remodelling (Pozzi et al., 2016; Frasch et al., 2015; Kilian et al., 2016). NAI-107 does not bind to the peptide stem, therefore these modifications seem not to play a role in the self-immunity mechanism of *Microbispora* sp. against this lanthipeptide, but may be involved in resistance against other PG-targeting antibiotics. However, the formation of tri/tripeptide and tri/tetrapeptide dimers linked by 3-3 bridges leads to balhimycin resistance *A. balhimycina* (Stegmann et al., 2015).

5. Conclusion

To understand the development of antibiotic resistance in pathogens, it is necessary to consider important reservoirs of resistance genes, which include determinants that confer self-resistance in antibiotic producing soil bacteria.

The main resistance of *A. balhimycina*, the GPA balhimycin producer, is based on the action of the VanHAX enzymes, resulting in the synthesis of PG precursors ending in D-Ala-D-Lac. It is supposed, that this resistance mechanism has been transferred to pathogens. Surprisingly, *A. balhimycina* is able to defend itself against GPAs even in the absence of the *vanHAX* genes. On the one hand, the strain is able to produce D-Ala-D-Lac ending precursors even in the absence of the canonical mechanism by using alternative routes, a resistance determinant that has not yet been identified in pathogens. On the other hand, the PG modifications on the precursors and the alternative cross-links confirm a “back up”-resistance mechanism, which occurs independently of the *vanHAX$_{Ab}$* genes. It is based on the elimination of pentapeptide stems ending in D-Ala$_5$ by the D,D-carboxypeptidases and the use of alternative transpeptidases (Ldt), leading to high level resistance to β-lactam antibiotics and to GPAs in enterococci. This type of modification has also been found in *Microbispora* sp., which does not produce any GPA. PG remodelling seems not only to be important for pathogenic bacteria; in fact, its impact in conferring competitive advantages in the natural environment can be speculated.

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