# Another Brick in the Wall: the role of the actinobacterial cell wall in antibiotic resistance, phylogeny and development

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# CHAPTER 3

# Substrate inhibition of VanA by D-alanine reduces vancomycin resistance in a VanX-dependent manner

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**ABSTRACT**

The increasing resistance of clinical pathogens against the glycopeptide antibiotic vancomycin, a last resort drug against infections with Gram-positive pathogens, is a major problem in the nosocomial environment. Vancomycin inhibits peptidoglycan synthesis by binding to the D-Ala-D-Ala terminal dipeptide moiety of the cell-wall precursor Lipid II. Plasmid-transferable resistance is conferred by modification of the terminal dipeptide into the vancomycin-insensitive variant D-Ala-D-Lac which is produced by VanA. Here we show that exogenous D-Ala competes with D-Lac as a substrate for VanA, increasing the ratio of wild-type over mutant dipeptide, an effect that was augmented by several orders of magnitude in the absence of the D-Ala-D-Ala peptidase VanX. LC-MS analysis showed that high concentrations of D-Ala led to the production of a significant amount of wild-type cell-wall precursors, while *vanX* null mutants produced primarily wild-type precursors. This enhanced the efficacy of vancomycin in the vancomycin-resistant model organism *Streptomyces coelicolor*, and the susceptibility of vancomycin-resistant clinical isolates of *Enterococcus faecium* (VRE) increased by up to 100-fold. The enhanced vancomycin sensitivity of *S. coelicolor* cells correlated directly to increased binding of the antibiotic to the cell wall. We isolated the VanX protein from *E.faecium* (VanB-type) and developed an assay with a fluorescent readout which can easily be adapted to a high throughput system for a potential inhibitor screen. Our work offers new perspectivesfor the treatment of diseases associated with vancomycin-resistant pathogens and for the development of drugs that target vancomycin resistance.

## INTRODUCTION

Infectious diseases caused by multi-drug-resistant (MDR) pathogens are spreading rapidly and are among the biggest threats to human health (Arias & Murray, 2009, Boucher *et al.*, 2009, Laxminarayan *et al.*, 2013, WHO, 2014). To deal with the increasing antibiotic resistance, novel antibiotics are called for, or alternatively the lifespan of the current drugs must be prolonged by compounds counteracting resistance. Exemplary is amoxillin-clavulanic acid (Augmentin), which is a combination of a β-lactam antibiotic (amoxilin), and a β-lactamase inhibitor (clavulanic acid) (Reading & Cole, 1977).

The cell wall and its biosynthetic machinery are a major target of the action of clinical antibiotics, including fosfomycin, bacitricin, cycloserine, β-lactam antibiotics (penicillins, cephalosporins) and glycopeptide antibiotics (vancomycin and teicoplanin) (Breukink & de Kruijff, 2006, Bugg *et al.*, 2011, Silver, 2013). Enterococci and many other Gram-positive pathogenic bacteria are resistant to a wide spectrum of antibiotics and can often only be treated with specific β-lactam antibiotics or with vancomycin (Rice, 2001, Frieden *et al.*, 1993, Bell *et al.*, 1998). Vancomycin resistance was first discovered in the 1950s (Walsh *et al.*, 1996). Vancomycin resistance is exchanged between bacteria via movable elements such as transposon *Tn1546*, which is carried by many vancomycin-resistant enterococci (VRE) (Courvalin, 2006). The most common forms of transferable vancomycin resistance are the VanA- and VanB-type resistance, the expression of which is inducible by vancomycin. VanA-type strains are resistant to high levels of vancomycin as well as to another glycopeptide antibiotic, teicoplanin, while VanB-type strains only show inducible resistance to vancomycin but retain susceptibility to teicoplanin (Aslangul *et al.*, 1997). While vancomycin resistance is most prevalent in enterococci (Murray, 2000), resistance has spread to methicillin-resistant *Staphylococcus aureus* (MRSA) (Howden *et al.*, 2010).

Vancomycin targets the cell wall and prevents cell growth by specifically binding to the D-alanyl-D-alanine (D-Ala-D-Ala) termini of the peptidoglycan (PG) precursor Lipid II, prior to its incorporation (Reynolds, 1989, Fischer *et al.*, 2013). The terminal D-Ala-D-Ala dipeptide is almost universally conserved in bacteria, the only exceptions being D-Ala-D-Lac or D-Alanyl-D-Serine in strains with either natural or acquired resistance to vancomycin (Vollmer *et al.*, 2008a). The VanA-type vancomycin resistance gene cluster in *S. coelicolor* consists of seven genes in four different operons: VanRS, VanJ, VanK and VanHAX that together mediate the substitution of the terminal D-alanine (D-Ala) by D-lactate (D-Lac), thereby decreasing the affinity of vancomycin for lipid II by three orders of magnitude (Walsh *et al.*, 1996, Smith *et al.*, 1999). The vancomycin resistance gene cluster provides resistance to both vancomycin and to teicoplanin and is located on the genome of the vancomycin producer *Amycolatopsis mediterranei* (Marshall *et al.*, 1998, van Wageningen *et al.*, 1998) as well as that of other actinomycetes, including the model species *Streptomyces coelicolor* A3(2) (Hong *et al.*, 2004).

Streptomycetes are Gram-positive soil bacteria with a complex multicellular life style (Claessen *et al.*, 2014, Flärdh & Buttner, 2009, Barka *et al.*, 2016). Streptomycetes are a major source of antibiotics and many other natural products of medical and biotechnological importance, such as anticancer, antifungal or herbicidal compounds (Bérdy, 2005, Hopwood, 2007). Due to the competitive environment of the soil, these microorganisms readily exchange genetic material, including antibi-

otic biosynthetic clusters and antibiotic resistance (Wiener *et al.*, 1998, Allen *et al.*, 2010). *S. coelicolor* is a non-pathogenic and genetically tractable model system for vancomycin resistance, with a well-annotated genome (Bentley *et al.*, 2002). The vancomycin resistance cluster of *S. coelicolor* consists of *vanRS,* a two-component regulatory system (TCS) consisting of sensory kinase VanS, and response regulator VanR that together ensure the transcription of the resistance genes in response to vancomycin challenge, and five resistance genes in the order *vanJKHAX*, with *vanHAX* forming a single transcription unit. Vancomycin resistant enterococci classically carry *vanRSHAX*, the function of which is highly similar to that in *S. coelicolor*, with the gene products VanH, VanA, VanX sharing 61%, 63% and, 64% aa identity, respectively, will the TCS components VanR and VanS share 31% and 25% aa identity, respectively (Hong *et al.*, 2004, Reynolds *et al.*, 1994). In response to vancomycin at the cell membrane, VanRS ensure the induction of the expression of *vanHAX,* and in the case of *S.coelicolor* also *vanK* and *vanJ* (Hutchings *et al.*, 2006). VanH produces D-Lac from pyruvate (Bugg *et al.*, 1991b), VanA is a D-alanyl-D-lactate (D-Ala-D-Lac) ligase (Wright & Walsh, 1992, Marshall *et al.*, 1997), VanX hydrolyses the D-Ala-D-Ala dipeptide and has been the target of previous studies assessing vancomycin sensitivity and resistance (Reynolds *et al.*, 1994, Wu *et al.*, 1995). VanX acts highly specific, it cleaves D-Ala-D-Ala, D-Ala-Gly, D-Ala-D-Ser, D-Ala-D-Val and D-Ala-D-Asn, but does not cleave D-Ala-L-Ala, L-Ala-D-Ala, L-Ala-L-Ala or the tripeptide D-Ala-D-Ala-D-Ala (Wu *et al.*, 1995). VanK attaches glycine to lipid II with D-Lac as the terminal residue (Hong *et al.*, 2004, Hong *et al.*, 2005). VanJ is not required for vancomycin resistance but is instead involved in the resistance to teicoplanin (Novotna *et al.*, 2012). Importantly, VanA is a bifunctional enzyme, which besides D-Ala-D-Lac can also produce the wild-type D-Ala-D-Ala dipeptide, although this is negligible during vancomycin challenge. (Hong *et al.*, 2005, Hong *et al.*, 2004, Verkade, 2008, Bugg *et al.*, 1991a). In this work, we show that D-Ala, but not L-alanine (L-Ala), acts as an inhibitor of the D-Ala-D-Lac ligase activity of VanA, an effect which is visible in the presence of vancomycin sensitive and resistant PG precursors. This effect was augmented by several orders of magnitude in *vanX* null mutants, effectively sensitizing the strains to vancomycin. We propose that a combination of D-Ala with a VanX inhibitor could resensitize clinical strains of VRE to vancomycin.

## MATERIALS AND METHODS

### Bacterial strains, culturing conditions and minimal inhibitory concentration (MIC)

*Escherichia coli* strains JM109 (Sambrook *et al.*, 1989) and ET12567 (Kieser *et al.*, 2000) were used for routine cloning procedures and for extracting non-methylated DNA, respectively. Cells of *E.coli* were grown in Luria-Bertani broth (LB) at 37°C. *Streptomyces coelicolor* A3(2) M145 was the parent of all mutants described in this work. All media and routine *Streptomyces* techniques were carried out as described (Kieser *et al.*, 2000). SFM (soy flour mannitol) agar plates were used for propagating *S. coelicolor* strains and to prepare spore suspensions. For liquid-grown cultures, *S. coelicolor* mycelia were grown in normal minimal media with phosphate (NMMP) supplemented with 1% (w/v) mannitol as the sole carbon source. The MIC of vancomycin against *S. coelicolor* M145 and its mutant derivatives were determined by growth on minimal media (MM) agar plates supplemented with 1% mannitol as the sole carbon source, and 0, 2, 4, 8, 16, 32, 64, 128, 256 or 512 $\mu g.ml^{-1}$ vancomycin, in combination with 0, 1, 5, 10 or 50 mM of D-Ala or L-Ala. Due to their much higher vancomycin sensitivity, *vanX* mutants were tested with 1, 5, 10, 50 and 100 $\mu$M D-Ala and L-Ala.

Five *vanA*-positive *Enterococcus faecium (E. faecium) strains* collected in 2011 and 2014 from patients in the Erasmus University Medical Centre, Rotterdam, The Netherlands were used. Presence of the *vanA* gene was confirmed by real-time PCR with the Light Cycler® 480 instrument (Roche Diagnostics, Almere, The Netherlands) with primers vanA F1 and vanA R1, and a *vanA*-specific probe labeled with 6-fluorescein amidite (FAM) at the 5' end and with black hole quencher (BHQ1) at the 3' end. The resistance profile of these isolates (Supplemental Table S2) was determined using the VITEK II (BioMerieux) system AST-P586. *To determine the MIC of vancomycin against E. faecium, cells* were grown overnight on Tryptic Soy Agar (TSA) blood agar plates (Becton Dickinson, Breda, The Netherlands) and suspended in 0.9% NaCl until $OD_{600}$ 0.5 (± 0.05). Of this suspension, 10 µl was dispensed into wells of sterile flat-bottom 96-well polystyrene tissue culture plates (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) containing serial dilutions of vancomycin in 190 µl of a 1:1 mixture of Fetal Bovine Serum (FBS) (Gibco, Bleiswijk, The Netherlands) and Iscove's Modified Dulbecco's Medium (IMDM) (without phenolred, Gibco, Bleiswijk, The Netherlands), and in the presence or absence of 50 mM D-alanine (Alfa Aesar, Ward Hill, MA, USA). Plates were incubated for 18-24 h at 37°C and MIC values determined visually following the CLSI guidelines, or by absorbance at 600 nm.

### VanX overexpression and isolation:

VanX was amplified from the genome of Enterococcus faecium (*vanB*-type) with primers pVanX_FW and pVanX_RV. The resulting product was inserted as a NdeI/BamHI fragment into pET28a resulting in vector: VanX-Pet28a. VanX-Pet28a was transformed to expression strain BL21(DE3) (fermentas) for protein expression. BL21(DE3) carrying VanX-Pet28a was grown in LB medium with 200$\mu$M $ZnSO_4$ and 50$\mu$g/ml kanamycin. For protein expression, the strain was grown at 30˚C to an OD of 1.8, at this point 1mM IPTG was added, biomass was harvested after 75 minutes(Wu *et al.*, 1995). The medium was centrifuged and the supernatant discarded. The pellet was frozen at -80˚C overnight. The following day the sample

was sonicated for 7.5 minutes in total. Cell debris was removed by several rounds of centrifugation (10.000g, 10 minutes ; 30.000g, 30 minutes ; 100.000g for 90 minutes). The supernatant was incubated overnight with Ni-column resin and purified the following day.

Buffer: PBS pH 7.4 + 200mM NaCl. Wash buffer: 20mM imidazole. Elution buffer: 200mM imidazole. The fractions were tested for activity by adding 5$\mu$L of each respective fraction to a reaction mix with 10mM Tris-HCl Ph7.0 with 100$\mu$M D-Ala-D-Ala. After an hour, 1$\mu$L from the reaction mix is spotted on a TLC.

**TLC conditions for separation of D-Ala-D-Ala and D-Ala.**

Samples were spotted on a silica TLC with D-Ala and D-Ala-D-Ala as controls. The running buffer was: n-butanol:acetic acid:water, 3:1:1. After the running buffer reached the top of the TLC plate, this was dried and afterwards stained with a Ninhydrin solution of: 0.1g Ninhydrin, 0.5mL acetic acid and 100mL acetone. After staining with ninhydrin, the plate was developed at 100 degrees for 5 minutes.

**Conditions VanX activity assay.**

The activity of VanX was measured by quantifying the amount of D-Ala generated by the dipeptidase activity. Each reaction contained: 20$\mu$M Amplex Red, 1$\mu$g/mL D-amino acid oxidase, 10$\mu$g/mL peroxidase and 100$\mu$M D-Ala-D-Ala (Arthur *et al.*, 1998). The reaction was read out in a TECAN Spark 10M plate reader with an excitation wavelength of 550 nm and a bandwidth of 20nm. The emission was measured at 590nm with a bandwidth of 20nm. The reaction needs to be prepared in the dark, UV light can catalyze the reaction from Amplex Red to resorufin.

**Constructs for gene disruption and complementation**

Deletion mutants were constructed according to a method described previously (Braun *et al.*, 2015). For deletion of *ddl*, the -948/+20 and +1173/+2638 regions relative to the translational start of *ddl* were amplified by PCR using primer pairs ddl_LF and ddl_LR, and ddl_RF and ddl_RR, using PCR conditions as described (Colson *et al.*, 2007). The left and right flanks were cloned into the multi-copy vector pWHM3 (Vara *et al.*, 1989), which is highly unstable in *Streptomyces* and therefore allows efficient gene disruption (van Wezel *et al.*, 2005). Subsequently, the apramycin resistance cassette *aac(3)IV* flanked by *loxP* sites was cloned into the engineered XbaI site to create deletion construct pGWS1152. The same strategy was used to create a construct for the deletion of *vanX*. In this case, the -1477/+30 and +572/+2035 regions relative to the start of *vanX* (SCO3596) were PCR-amplified using primer pairs vanX_LF and vanX_LR, and vanX_RF and vanX_RR (Supplemental table S3). Insertion of *aac(3)IV-loxP* site in the engineered XbaI site generated deletion construct pGWS1164. The presence of *loxP* sites allows the efficient removal of the apramycin resistance cassette from the chromosome following the introduction of plasmid pUWLCRE that expresses the Cre recombinase (Fedoryshyn *et al.*, 2008).

**Complementation constructs**

A construct for the genetic complementation of *ddl* was made by amplifying the promoter- and coding region of *ddl* using primers ddlcomp_F and ddlcomp_R (nt positions: -573/+1184 relative to the start of *ddl* (Supplemental table S3), and inserted as an EcoRI/BamHI fragment in the low copy vector pHJL401 (Larson & Hershberger, 1986), a highly stable low-copy number vector that is well suited for genetic

complementation (van Wezel *et al.*, 2000b), resulting in pGWS1159.

**Fluorescence microscopy**

Samples were grown for 18 h in liquid NMMP after which a sample was taken from the culture to stain with BODIPY FL vancomycin (Vanco-FL) as described (Daniel & Errington, 2003). Equal amounts of unlabeled vancomycin and Van-FL were added to the sample to a final concentration of 1 $\mu$g/ml and was incubated for 10-20 min at 30°C. Directly after taking the first sample, 50 mM D-Ala was added to the medium and was left to grow for another hour before imaging the effect of added D-Ala. Imaging was done as described previously (Willemse & van Wezel, 2009). A Zeiss observer with a Plan-Neofluar 40x/0.9 lens was used, and GFP was excited with a wavelength of 488 nm and observed at 515 nm with filter BP505-550, with illumination power set to 7.5%. The images were analyzed with ImageJ, all the fluorescent images were processed identically. The final figure was made with Adobe Photoshop CS6.

**Isolation of cytoplasmic peptidoglycan precursors**

For the cytoplasmic PG precursor isolation and identification we used a modification of the method described previously by Hong and colleagues (Hong *et al.*, 2004). Where applicable, 10 $\mu$g vancomycin was added to the strains at the moment of inoculation. The strains were grown in NMMP (1% (w/v) Mannitol, 50 mM $MgCl_2$) until mid-log phase (OD-0.3-0.4) and mycelia were harvested by centrifugation at 4°C and washed in 0,9% NaCl. Mycelia was extracted with 5% cold trichloric acid (TCA) for 30 minutes at 4°C. This was centrifuged and the supernatant desalted on a Sephadex G-25 column (Illustra NAP-10 Columns, GE Healthcare, Pittsburgh), and concentrated by rotary evaporation. The concentrated precursors were dissolved in HPLC-grade water and separated by LC-MS using a gradient of 0-20% acetonitrile in water with 0,1% TFA. The elution was monitored at absorbance 254 nm and by the sizes eluted (1193.8 - 1195.3 m/z).

For the measurement over time, the protocol was adjusted in the following way: 300 ml NMMP cultures were grown until exponential phase (OD 0.3-0.4), at which point a 10 ml sample was taken (sample t=0) and 50 mM of D-Ala or L-Ala was added to the original culture, followed by further sampling after 1, 5, 15, 30, 60, 120 and 180 minutes. Samples were rapidly filtered with a vacuum pump and washed with 0.9% (w/v) NaCl, mycelia scraped off the filter and transferred to 5% TCA.

## RESULTS

### D-Ala reduces vancomycin resistance

The bifunctional activity and structural analysis of the VanA enzyme implies that it can use both D-Lac and D-Ala as a substrate (Roper *et al.*, 2000, Wright & Walsh, 1992), suggesting that D-Ala might be able to compete with D-Lac in the active site of the enzyme. To test the applicability of this concept, we used the naturally vancomycin-resistant *S. coelicolor* M145 as a model system. The strain was grown on minimal media (MM) agar plates with increasing concentrations of D-Ala and vancomycin. D-Ala was thereby added in concentrations of 5, 10 or 50 mM and the effect on the MIC of vancomycin assessed. As controls we added either L-Ala or neither alanine stereoisomer. In the absence of added amino acids, the MIC of vancomycin against *S. coelicolor* was 128 μg/ml. Supplementing the media with up to 50 mM L-Ala did not have any effect on the susceptibility to vancomycin (Table 1 and Fig S1). Sensitivity to vancomycin increased significantly when D-Ala was added; at 10 mM D-Ala, the MIC decreased to 32 μg/ml (4-fold reduction), while at 50 mM D-Ala the MIC was reduced to 4 μg/ml (32-fold reduction) (Table 1 and Fig S1). This supports the concept that D-Ala can reduce VanA-based vancomycin resistance, presumably by competing with the substrate D-Lac at the active site of the VanA enzyme (Arthur *et al.*, 1994, Zarlenga *et al.*, 1992).

**Table 1.** Effect of D-Ala on the MIC of vancomycin (in μg/ml) against *S. coelicolor* M145 and its mutant LAG2.

| | No AA | D-Ala | | | L-Ala | | |
|---|---|---|---|---|---|---|---|
| Strain | | 5 mM | 10 mM | 50 mM | 5 mM | 10 mM | 50 mM |
| M145 | 128 | 32 | 32 | 4 | 128 | 128 | 128 |
| Δ*ddl*[a] | 128 | 32 | 32 | 4 | 128 | 128 | 128 |
| LAG2 | 128 | 64 | 32 | 4 | 128 | 128 | 128 |

[a]. The *ddl* null mutant is not viable on medium without vancomycin but furthermore had an identical MIC value to M145.

### Creation of a vancomycin-independent *ddl* mutant

To study the molecular basis of this effect in more detail, a strain was required that depends on *vanA* for the synthesis of the D-Ala-D-Ala dipeptide and thus for cell-wall synthesis. The wild-type gene for D-Ala-D-Ala ligase is *ddl* (SCO5560 in *S. coelicolor*), which is essential for normal growth, but it's absence can be rescued by the vancomycin-inducible expression of *vanA,* the only other paralogue of *ddl* on the *S.coelicolor* genome (Wright & Walsh, 1992, Kwun *et al.*, 2013). To allow direct comparison with other mutants related to GlcNAc and cell-wall metabolism previously made in our laboratory (Nothaft *et al.*, 2010, Rigali *et al.*, 2008, Swiatek *et al.*, 2012), a *ddl* (SCO5560) null mutant was created in our specific *S. coelicolor* M145 laboratory host, thereby ensuring that all of the mutants have the same isogenic background. This was done by replacing the entire *ddl* coding region by the apramycin resistance cassette (*aacC4)* via homologous recombination and subsequent removal to leave an in-frame deletion of *ddl* in the genome. The *aacC4* gene was flanked by *loxP* sites, allowing the subsequent removal by expression of the Cre recombinase, resulting in a markerless deletion mutant of *ddl* (see Materials and

Methods section). To compensate for the absence of D-Ala-D-Ala, the *ddl* mutant was created in the presence of vancomycin, so as to elicit the production of the alternative precursor dipeptide D-Ala-D-Lac by VanA (Hong *et al*., 2005). Many candidate *ddl* null mutants were obtained, all of which failed to grow in the absence of vancomycin and showed normal sporulation. One of these strains was selected for further characterization. The absence of *ddl* in this mutant was confirmed by PCR (data not illustrated). Expectedly, the *ddl* mutant could only grow on agar plates with vancomycin (Fig. 1). Introduction of plasmid pGWS1159, which expresses the *ddl* gene from its own promoter, into the *ddl* null mutant restored normal development and growth in the absence of D-Ala (data not illustrated).

To allow studying the sensitivity of VanA to inhibitory molecules regardless of the presence or absence of vancomycin, we selected for suppressor mutants by plating spores ($10^7$ cfu) of the *ddl* null mutant onto SFM agar plates lacking vancomycin, so as to select for suppressors with constitutive expression of the vancomycin resistance cluster. This yielded a small number of spontaneous suppressor mutants, which occurred at a frequency of around $10^{-6}$. One of these mutants has been selected and designated LAG2. These constitutively expressed the vancomycin resistance cluster, as this is a requirement to compensate for the absence of *ddl*. One of the suppressor mutants was selected and designated LAG2 (Fig. 1)

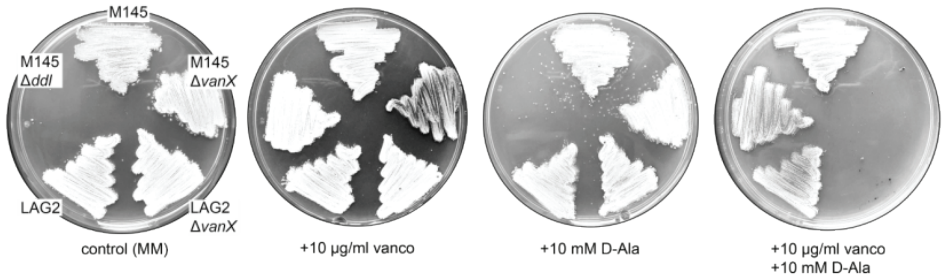


**Figure 1. Effect of D-Ala on growth of *S. coelicolor* M145 and derivatives.** Strains are *S. coelicolor* M145 (parental strain), its *vanX* mutant, suppressor mutant LAG2, LAG2 Δ*vanX* and M145 Δ*ddl.* Strains were streaked on MM with from left to right, no additives (control); MM with 10 µg/ml vancomycin; 10 mM D-Ala; 10 µg/ml vancomycin + 10 mM D-Ala. The *ddl* mutant fails to grow in the absence of vancomycin, a phenotype that is suppressed in LAG2 due to constitutive expression of the *van* resistance cluster. Note the high sensitivity of the *vanX* null mutants of M145 and LAG2 to the combination of vancomycin and D-Ala. Plates were incubated for 3 days at 30˚C.

DNA sequencing of the vancomycin resistance genes *vanRSJKHAX* of strain LAG2 revealed that the insertion element IS466A (SCO3469) (Yamasaki *et al*., 2000, Hong *et al*., 2008, Courvalin, 2006) had inserted at nt position 55 relative to the translational start of *vanS,* causing loss-of-function. This spontaneous integration event in *vanS* had been observed before in both *Streptomyces* and in strains of *Enterococcus*, and results in constitutive upregulation of the vancomycin resistance cluster (Hutchings *et al*., 2006, Thaker *et al*., 2015, Arthur *et al*., 1997). The *ddl* suppressor mutant LAG2 had a similar level of vancomycin resistance as the parental strain, with an MIC of 128 µg/ml (Table 1). Similar as seen for wild-type cells, addition

of L-Ala did not affect the MIC for vancomycin, while addition of D-Ala decreased the MIC to 4 μg/ml when 50 mM D-Ala was added to the agar plates (Table 1). Thus, while LAG2 constitutively expresses the vancomycin resistance cluster, it has a comparable MIC to vancomycin as wild-type cells, which in both cases could be strongly reduced by the addition of D-Ala.

**Deletion of *vanX* amplifies the effect of D-Ala on vancomycin sensitivity**

We then wondered if targeting *vanX* could further potentiate the effect of D-Ala as inhibitor of vancomycin resistance. VanX hydrolyzes D-Ala-D-Ala, thereby counteracting the accumulation of wild-type precursors and supporting vancomycin resistance (Lessard & Walsh, 1999, Tan *et al.*, 2002). A *vanX* null mutant was created using a similar strategy as for *ddl*, replacing the coding region of *vanX* by the apramycin resistance cassette *aacC4*. The mutant was created in both the parental strain *S. coelicolor* M145 and in its *ddl* suppressor mutant LAG2, generating M145 Δ*vanX* and LAG4 (LAG2 Δ*vanX*), respectively.

The respective *vanX* mutants of M145 and LAG2 grew on medium supplemented with 10μg/ml vancomycin, and 10 mM D-Alanine but failed to grow on media containing both vancomycin and D-Alanine at a concentration where M145 and LAG2 did not show sensitivity to vancomycin (Figure 1). LAG2Δ*vanX* produced 20% wild-type precursors prior to the addition of D-Ala. This strongly suggests that VanA produces a significant amount of D-Ala-D-Ala *in vivo*, which accumulates in the absence of VanX. Consistent with this idea, the MIC of vancomycin was lower for the *vanX* mutant, namely 32 μg/ml for the *vanX* mutant, and 64 μg/ml for LAG2Δ*vanX*, as compared to 128 μg/ml for the parental strain M145 (Table 2)

In wild-type cells, 50 mM D-Ala was required to reduce the MIC for vancomycin to 4 μg/ml. However, only 50 μM D-Ala was required to reduce the MIC of vancomycin for the *vanX* mutant to 1 μg/ml. This spectacular difference means that D-Ala is around 4000 times more effective in the absence of the D-Ala-D-Ala peptidase activity of VanX. This is consistent with the very strong accumulation of wild-type precursors in *vanX* null mutants as compared to the *vanX*-positive parental strain.

**Table2.** The MIC of vancomycin (μg/ml) on *S.coelicolor* vanX-mutants with D-Ala.

| | **No AA** | **D-Ala** | | | **L-Ala** | | |
|---|---|---|---|---|---|---|---|
| **Strain** | | **10 μM** | **50 μm** | **100 μM** | **10μM** | **50μM** | **100μM** |
| M145ΔvanX | 32 | 16 | 1 | 1 | 32 | 32 | 32 |
| LAG2ΔvanX | 64 | 32 | 8 | 2 | 32 | 32 | 32 |

**Analysis of peptidoglycan precursors**

In order to get more insight into the synthesis of vancomycin-sensitive (*i.e.* wild-type) or vancomycin-resistant PG, the pool of PG precursors was analyzed by Liquid Chromatography coupled to Mass Spectrometry (LC-MS) (Arthur *et al.*, 1994, Arthur *et al.*, 1998, Kwun *et al.*, 2013) When cells produce wild-type PG, only MurNAc-pentapeptides with a D-Ala-D-Ala terminus are detected, while vancomycin-resistant PG precursors have a D-Ala-D-Lac terminus. Wild-type precursors ending with D-Ala-D-Ala are characterized by a peak with a monoisotopic mass of 1994 Da and a retention time of around 7.2 minutes, while vancomycin-insensitive precursors ending with D-Ala-D-Lac are characterized by a peak of a monoisotopic mass of 1995 Da and a significantly higher retention time of around 8.2 minutes (Fig. 3A).

In extracts from the parental strain grown in the absence of vancomycin, only wild-type precursors were observed (Fig. 3B). Expectedly, when *S. coelicolor* M145 was grown in the presence of 10 $\mu$g/ml vancomycin, the vast majority of the precursors (91.5%) represented the vancomycin-insensitive variant. Similarly, 95.7% of the precursors from the *ddl* null mutant grown in the presence of vancomycin contained the terminal D-Ala-D-Lac dipeptide (Fig. 3B). This indicates that VanA produces a low level of the D-Ala-D-Ala dipeptide. In the *ddl* suppressor mutant LAG2, which constitutively expresses the vancomycin resistance gene cluster, nearly all PG precursors terminated with D-Ala-D-Lac (99.8% and 99.7% for cultures grown with and without vancomycin, respectively) (Fig. 3B). We then wondered how D-Ala would affect the accumulation of wild-type precursors over time in the suppressor mutant. The constitutive expression of the vancomycin resistance cluster in the suppressor mutant allows growth of *ddl* null mutants without the need for vancomycin and ensures that the result is caused by substrate competition, not a difference in the expression of the vancomycin resistance cluster. For the time-lapse experiment, 300 ml NMMP cultures were supplemented with either D-Ala or L-Ala (control) at 50 mM end concentration, and 10 ml samples were collected prior to and 1, 5, 15, 30, 60, 120 and 180 min after the addition of either alanine stereoisomer. Prior to the addition of D-Ala or L-Ala (t=0), LAG2 did not accumulate any wild-type precursors. However, addition of 50 mM D-Ala resulted in the production of small amounts of wild-type precursor (1%) within 1 min. After 15 min this amount had increased to 4%, which appeared to be close to the maximum, with levels of wild-type precursors never exceeding 5%. L-Ala did not result in detectable levels of wild-type precursors in LAG2.

Strikingly, analysis of PG precursors in *vanX* null mutants revealed that the addition of even low levels of D-Ala facilitated the accumulation of high levels of wild-type precursors, up to as much as 80% wild-type precursors 3 h after the addition of D-Ala (Figure 3). This supports the notion that in the absence of VanX, wild-type precursors are incorporated much more frequently into the cell wall, with increased sensitivity to vancomycin as a consequence.

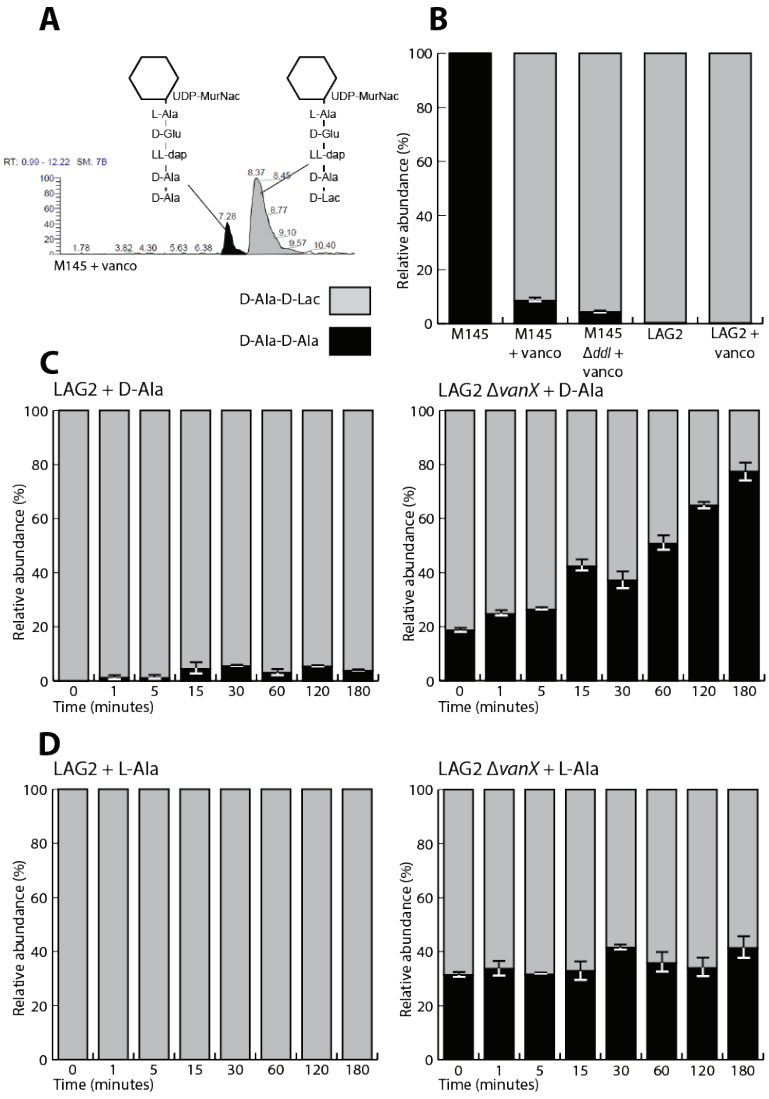


**Figure 2. LC-MS analysis of peptidoglycan precursors.** (A) Example peak profile and corresponding precursors of *S. coelicolor* M145 grown with vancomycin, with the peak area corresponding to a precursor terminating in D-Ala-D-Ala shown in black, and the peak corresponding to a precursor terminating in D-Ala-D-Lac in grey. (B) Ratio (in %age) of wild-type (black) and vancomycin-resistant (grey) precursors in *S. coelicolor* M145, its *ddl* null mutant and suppressor mutant LAG2, grown with or without vancomycin (10 μg/ml). The *ddl* mutant is only shown with vancomycin as it fails to grow in its absence. LAG2 with and without vancomycin have less than 1% wild-type (vancomycin-sensitive) peptidoglycan. Strains were grown with or without vancomycin until OD 0.3-0.4 before harvesting.(C) Accumulation of wild-type and vancomycin-resistant precursors over time in LAG2 and LAG2Δ*vanX*. The samples were grown until OD 0.3-0.4 prior to the addition of 50 mM D-Ala. Samples were taken prior to (0) or 1, 5, 15, 30, 60, 120 or 180 minutes after the addition of D-Ala. (D) Same as (C), but with L-Ala instead of D-Ala.Bars representing the precursors shown as percentages (total set to 100%).

**Visualization of vancomycin binding by fluorescence microscopy**

To qualitatively determine the ability of vancomycin to bind to the cell walls of different *Streptomyces* strains and also visualize the effect of D-Ala, mycelia of *S. coelicolor* were fluorescently stained with BODIPY-FL vancomycin (Vanco-FL). In vancomycin-sensitive bacteria, vancomycin localizes in foci at sites of *de novo* cell wall synthesis (Daniel & Errington, 2003). In *S. coelicolor*, which grows by tip extension (Gray *et al.*, 1990), these sites are in particular the hyphal tips and cell division septa.

While hyphae of *S. coelicolor* M145 were stained well by Vanco-FL, hardly any Vanco-FL bound to the hyphae of strains constitutively expressing vancomycin resistance (LAG2 or LAG2Δ*vanX*) (Fig. 4A). However, addition of D-Ala resulted in marginal staining by Vanco-FL of the LAG2 hyphal tips (Fig. 4B); in contrast, its *vanX* mutant derivative LAG4 was stained very well, in line with the strongly enhanced vancomycin sensitivity of the mutant (Fig. 4).

Taken together, our mutational, microscopy and LC-MS experiments show that D-Ala effectively and specifically enhances the sensitivity of vancomycin resistant *S. coelicolor* to vancomycin, by allowing accumulation of wild-type cell-wall precursors and thus binding of vancomycin to sites of active cell-wall biosynthesis. This effect was strongly enhanced in *vanX* mutants (which lack D-Ala-D-Ala peptidase activity).

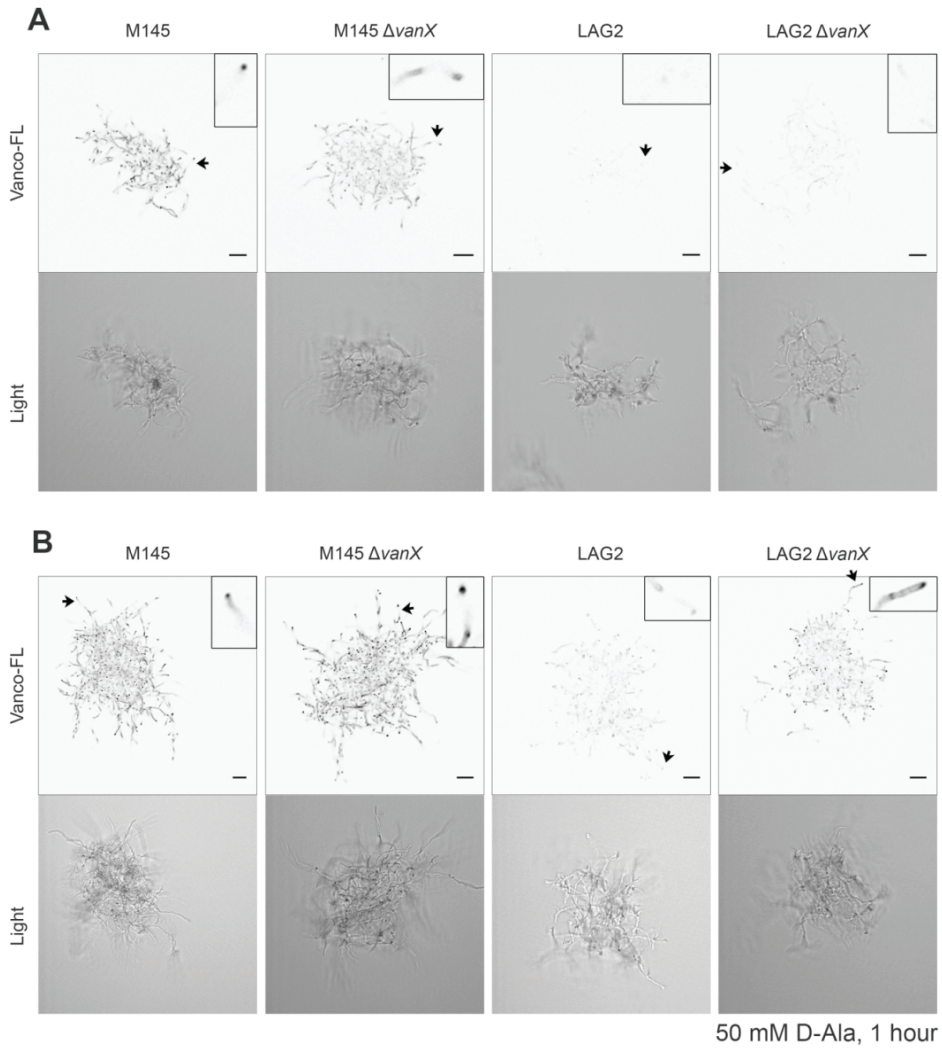


**Figure 3. Fluorescence micrographs of Vanco-FL stained hyphae.** To analyze vancomycin binding, *S. coelicolor* strains M145, M145 Δ*vanX*, LAG2 and LAG2 Δ*vanX*, were grown in liquid NMMP for 12 h, and continued to grow for 1 h in the absence (A) or presence (B) of 50 mM D-Ala. Mycelia were then stained with Vanco-FL and imaged. Top, fluorescence micrographs (inverted greyscale); bottom, corresponding light images. *S. coelicolor* M145 and its *vanX* mutant were readily stained by Vanco-FL. Constitutively vancomycin resistant strain LAG2 was not stained by Vanco-FL in the absence of D-Ala, and showed some binding after the addition of D-Ala. Extensive Vanco_FL staining was seen for LAG2Δ*vanX* only after the addition of D-Ala. Inserts are magnifications of the areas indicated by arrows in the respective image. Scale bar, 10 μm.

**Analysis of the effect of D-Ala on the MIC of clinical isolates of VRE**

Having established that D-Ala enhances the efficacy of vancomycin against vancomycin-resistant *S. coelicolor*, we then assessed its effect on the resistance of *vanA*-positive clinical isolates of *E. faecium*. MIC values were calculated by testing a serial (two-fold) dilution of vancomycin in the presence or absence of D-Ala in triplicate (Table 3). ]Similar as seen for *S. coelicolor,* addition of 50 mM D-Ala to the growth media resulted in a strong increase in the efficacy of vancomycin against all clinical isolates, with reduction of 4-7 dilution steps. Even in the worst cases, the MIC of vancomycin was still reduced 16-32 fold (from 4096 μg/ml to 256 μg/ml or 128 μg/ml), while we also noted a further decrease to values as low as 16 μg/ml for strain vanA10. This value corresponds to intermediate resistance.

**Table 3.** MIC of vancomycin (μg/ml) against VRE in the presence or absence of D-Ala.

| Strain | **0 mM D-Ala** | **50 mM D-Ala** | **steps down** |
|---|---|---|---|
| vanA1 | 4096 | 256 | 4 |
| vanA2 | 4096 | 256 | 4 |
| vanA3 | 4096 | 128 | 5 |
| vanA4 | 4096 | 128 | 5 |
| vanA10 | 2048 | 16 | 7 |

**VanX isolation and characterization.**

Here, we have found that VanX is an essential protein in the vancomycin resistant complex, a knockout of the *vanX* gene in *S.coelicolor* causes it to become a thousand fold more sensitive to vancomycin when combined with D-Ala. For this reason, we expressed and isolated the VanX protein from *Enterococcus faecium* and developed an assay with a fluorescent readout. VanX is a 23.35Da protein with dipeptidase activity which is highly specific for D-Ala-D-Ala, it does not cleave the stereoisomer L-Ala-D-Ala or L-Ala-L-Ala. The binding pocket for D-Ala-D-Ala is located in the center of the protein and requires a $Zn^{2+}$ cofactor (Figure 5). The protein was amplified from the genome of a VanB-type *Enterococcus faecium*, this was cloned into pET-28a, which carries a HIS-tag at the N-terminus and expressed in *E.coli* BL21 (DE3). The protein was purified using a nickel column and the activity was initially tested by adding 5$\mu$L of the fraction to a 20$\mu$L reaction mix in 10mMTris-HCl, pH 7.0 and 100$\mu$M D-Ala-D-Ala. After an hour incubation, the mix is spotted on a silica TLC with running buffer n-butanol:acetic acid:water, 3:1:1, until the solution almost hit the top of the TLC plate. The plate was developed with a ninhydrin stain. this showed that the protein was still active after purification.

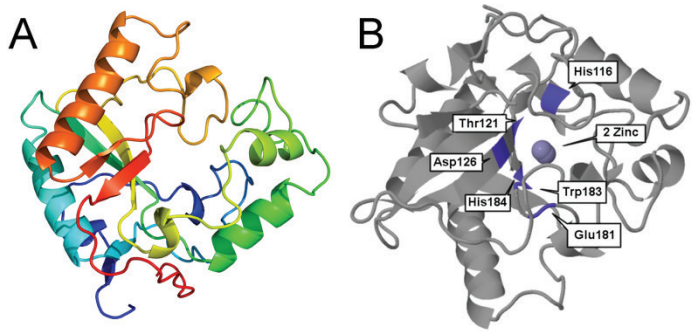


**Figure 5.** (A) Structural model of VanX. PHYRE²(Kelley *et al.*, 2015) model of VanX, The N- to C-terminus is rainbow-coloured. (B) The D-Ala-D-Ala binding site prediction, a small pocket of amino acids makes up the active site, associated with a $Zn^{2+}$ cofactor.

To measure the activity of VanX, it is neccesary to differentiate between D-Ala and D-Ala-D-Ala. Although this is straightforward by TLC, this method would not be feasible for a high-throughput assay. An assay with a fluorescent output was developed based on the activity of a D-amino acid oxidase (DAO), this protein oxidases D-Ala, but not D-Ala-D-Ala. CAO converts D-Ala to pyruvate, ammonia and hydrogen peroxide, after which Horseradish peroxidase catalyzes the reaction of hydrogen peroxide and Amplex Red to the fluorescent Resorufin and oxygen, as shown in Figure 6. The reaction without VanX (0 VanX) showed a flat slope, the reaction with D-Ala instead of D-Ala-D-Ala showed high initial fluorescence and bleached afterwards. An increasing amount of VanX (5-50 $\mu$g/ mL) increased the primary reaction speed, from 10$\mu$g/mL on, the slope was the same, indicating that another protein is the limiting step. With these concentrations, 5-10 $\mu$g VanX would be ideal for a high-throughput set up.

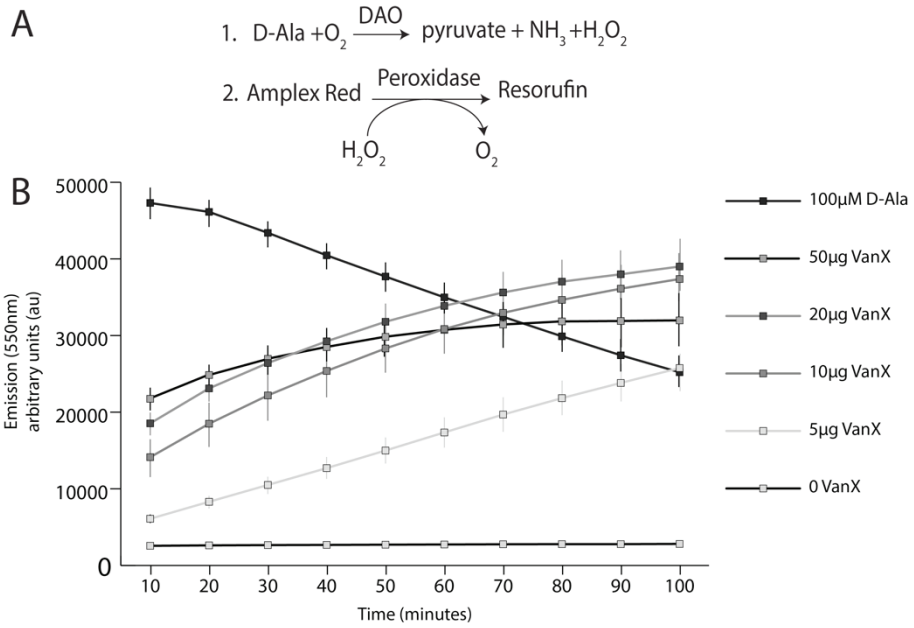


**Figure 6.** A: The fluorescence assay is based on two reactions: 1. The oxidation of D-Ala by DAO which gives pyruvate, ammonia and hydrogen peroxide. 2. Hydrogen peroxide is then converted to oxygen by horseradish peroxidase for which Amplex red is the electron donor converting that into Resorufin which gives a strong fluorescent signal. B: Fluorescence assay to measure D-Ala, the plate was measured every ten minutes, 5 wells are averaged for every data point. The y-axis shows the emission at 550 nm, measured in arbitrary units (au). The x-axis shows time in minutes. The reaction was performed with 0, 5, 10, 20 and 50$\mu$g/mL VanX protein and with 100$\mu$M D-Ala as a control.

## DISCUSSION

With the rapid spread of vancomycin resistance, new efforts are needed to maintain this last resort antibiotic as a clinical drug against multi-drug resistant bacterial infectious diseases. So far, attempts included engineering VanX-inhibitors (Muthyala *et al.*, 2014, Wu *et al.*, 1995, Aráoz *et al.*, 2000), or re-engineering vancomycin itself to target not only the cell-wall precursors with D-Ala-D-Ala termini, but also those ending with D-Ala-D-Lac (Xie *et al.*, 2011). As a basis to develop new approaches to target vancomycin resistance, we studied the model organism *S. coelicolor*, which has a very similar set of vancomycin resistance genes as the pathogenic VRE (Wiener *et al.*, 1998).

VanA, a variant of Ddl that ligates D-Ala to D-Lac to form D-Ala-D-Lac, plays a key role in vancomycin resistance. VanA is a bifunctional enzyme which can produce both D-Ala-D-Lac and D-Ala-D-Ala, the affinity for either D-Lac or D-Ala as a substrate being highly dependent of substrate and pH (Verkade, 2008, Healy *et al.*, 2000, Lessard *et al.*, 1999). The extracellular addition of high concentrations D-Ala results in increased accumulation of wild-type cell-wall precursors and consequently the build-up of vancomycin-sensitive PG, due to competition with D-Lac at the active site of VanA, while supplementing D-Lac leads to a high abundance of precursors terminating in D-Ala-D-Lac (Kwun *et al.*, 2013, Arthur *et al.*, 1998, Arthur *et al.*, 1994) This effect is specific for vancomycin and did not work on A40926, the natural product of the novel glycopeptide dalbavancin, of which the mechanism is similar but different from vancomycin. We analyzed the accumulation of precursors, which revealed that supplementing cultures of a constitutively vancomycin-resistant variant of *S. coelicolor* M145 with excess D-Ala, resulted in accumulation of up to 5% wild-type precursors. While interesting, the effect is too low to be effective in treatment of vancomycin-resistant pathogens. However, we here show that the effect of D-Ala is massively enhanced in the absence of VanX, with up to 80% of the precursors accumulated in *vanX* null mutants containing the wild-type dipeptide. As support of the biochemical data, active incorporation of wild-type precursors at apical sites was visualized with Vanco-FL, which fluorescently stains all sites of active cell-wall synthesis, *i.e.* the hyphal tips and newly synthesized septa. While wild-type cells and *vanX* mutant cells were stained very well by Vanco-FL, derivatives with constitutive vancomycin resistance were hardly stained. However, addition of D-Ala recovered fluorescence even to cells with constitutive vancomycin resistance, indicative of the incorporation of wild-type cell-wall material concomitant with increased sensitivity to vancomycin. Other ways D-Ala could affect vancomycin sensitivity could be by D,D-transpeptidases in the periplasm substituting D-Lac for D-Ala on the precursors (Hugonnet *et al.*, 2014). The strong direct correlation between PG precursor accumulation and vancomycin binding (as determined by imaging fluorescent vancomycin) argues against a major influence of D,D-transpeptidases in this work. The fluorescence correlated with the level of wild-type cell-wall precursors in the various strains, and this method therefore offers rapid qualitative assessment of vancomycin sensitivity, which could be applied in high-throughput screening for compounds that potentiate vancomycin resistance. By combining the precursor analysis and staining with Vanco-FL it is also clear that D-Ala is not only incorporated in PG precursors but the pentapeptides terminating in D-Ala-D-Ala are displayed at the cell surface. A question which remains though is which amount of vancomycin sensitive PG would be sufficient to regain sensitivity against vancomycin.

Previous work indicated that the deletion of *vanX* increases the sensitivity to vancomycin (Arthur *et al.*, 1998). However, as our work shows, significant changes in the MIC are only brought about when D-Ala is added as competitive inhibitor for D-Lac. This change in response to the deletion of *vanX* may well depend on the target organism, which is underlined by the differential effect of added D-Ala on the MICs of independent clinical VRE isolates. Based on the findings presented in this work, we propose a model for vancomycin resistance in which the catalytic activity of VanA depends largely on the available substrate (Fig. 5). In the presence of excess of D-Ala, VanA is bifunctional and synthesizes both D-Ala-D-Ala and D-Ala-D-Lac, but the wild-type dipeptide is then cleaved by the VanX peptidase. However, excess of D-Ala will result in such large amounts of D-Ala-D-Ala that VanX cannot degrade the dipeptides sufficiently rapidly to avoid their use as substrate by VanA, thus resulting in low levels of wild-type lipid II. As a result, a small proportion of wild-type PG is produced, giving enhanced vancomycin sensitivity. In the absence of *vanX* the addition of even very small amounts of D-Ala (10-50 $\mu$M instead of 10-50 mM) already led to strong accumulation of wild-type precursors and a drop in the MIC of vancomycin to values as low as 1 $\mu$g/ml. This is well within the range of clinical sensitivity.

How can the concepts developed in this work be implemented into approaches to counteract vancomycin resistant Gram-positive pathogens like VRE and VRSA? The high sensitivity of *vanX* null mutants to the combination of vancomycin and D-Ala strongly suggests that the combined treatment with vancomycin and D-Ala will be particularly effective in combination with molecules that perturb the bioactivity of VanX. VanX inhibitors have been described in the literature, but their effect was limited (Muthyala *et al.*, 2014, Chang *et al.*, 2006, Aráoz *et al.*, 2000, Yang *et al.*, 2011b, Wu *et al.*, 1995). Based on the data presented here, this is likely explained by the fact that the effect of a *vanX* deletion without additional D-Ala is very limited, only decreasing the MIC by two-fold in this work. Similarly, the data also point out that VanX inhibitors that have been or will be developed in the future should be (re)tested in the presence of added D-Ala, as this largely augments their efficacy. Strains that depend on the vancomycin resistance cluster for growth thereby candidate as screening hosts for a high-throughput screen of small molecules that target vancomycin resistance. This may prove an important asset in the hunt for drugs that counteract vancomycin-resistant pathogens such as VRE and VRSA.

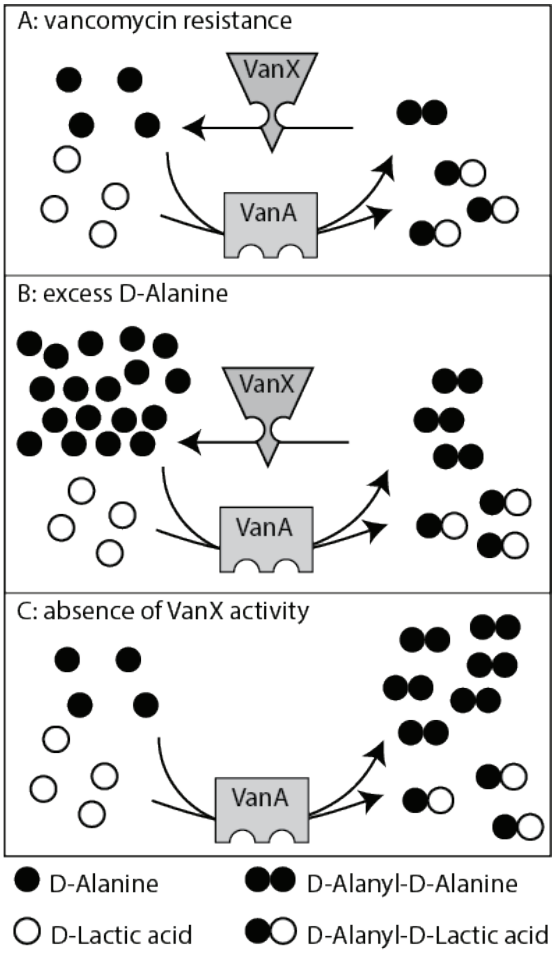


**Figure 4. Model of how D-Ala influences the activity of VanA in the presence or absence of VanX.** All drawings show the situation where VanA is constitutively expressed and in the absence of Ddl. (A) Normal situation. VanA produces both D-Ala-D-Ala and D-Ala-D-Lac, whereby D-Ala-D-Ala is broken down by VanX (resulting in a strong bias for D-Ala-D-Lac). (B) situation in the presence of excess D-Ala, which is then preferentially used as substrate by VanA to favor the formation of the D-Ala-D-Ala dipeptide, which is however still broken down by VanX. (C) situation in the absence of *vanX*. Because of the lack of VanX activity, D-Ala-D-Ala accumulates and the pool of D-Ala-D-Ala is dramatically increased when access D-Ala is added. This then enhances the percentage of wild-type cell-wall precursors and strongly amplifies the efficacy of vancomycin.

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**Authors contributions**

LvdA: Conception and design, performed the work on *S.coelicolor*, wrote and revised the article. NL: performed the work on *E. faecium*. WvW designed the work on *E. faecium* and wrote the article. GvW: Conception and design, wrote and revized the article. All authors read and agreed on the final version of the article.