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Substrate Inhibition of VanA by D-Alanine Reduces Vancomycin Resistance in a VanX-Dependent Manner

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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 to mutant dipeptide, an effect that was augmented by several orders of magnitude in the absence of the D-Ala–D-Ala peptidase VanX. Liquid chromatography-mass spectrometry (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. Our work offers new perspectives for the treatment of diseases associated with vancomycin-resistant pathogens and for the development of drugs that target vancomycin resistance.

Infectious diseases caused by multidrug-resistant (MDR) pathogens are spreading rapidly and are among the biggest threats to human health (1–4). A particular problem with drug discovery from microbial sources is the high frequency of rediscovery of known compounds, which necessitates new approaches to replenish the antimicrobial drug pipelines (5–7). To deal with the increasing antibiotic resistance, novel antibiotics are called for, or alternatively, the life spans of the current drugs must be prolonged by compounds counteracting resistance. Exemplary is amoxicillin-clavulanic acid (Augmentin), which is a combination of a β-lactam antibiotic (amoxicillin) and a β-lactamase inhibitor (clavulanic acid) (8).

The cell wall and its biosynthetic machinery are a major target of the action of clinical antibiotics, including fosfomycin, bacitracin, cycloserine, β-lactam antibiotics (penicillins and cephalosporins), and glycopeptide antibiotics (vancomycin and teicoplanin) (9–11). Enterococci and many other Gram-positive pathogenic bacteria are resistant to a wide spectrum of antibiotics and can often be treated only with specific β-lactam antibiotics or with vancomycin (12–14). Vancomycin resistance was first discovered in the 1950s (15). Vancomycin resistance is exchanged between bacteria via movable elements such as transposon Tn*1546*, which is carried by many vancomycin-resistant enterococci (VRE) (16). 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 show only inducible resistance to vancomycin but retain susceptibility to teicoplanin (17). While vancomycin resistance is most prevalent in enterococci (18), resistance has spread to methicillin-resistant *Staphylococcus aureus* (MRSA) (19).

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 (20, 21). The terminal D-Ala–D-Ala dipeptide is almost universally conserved in bacteria, with the only exceptions being D-Ala–D-Lac or D-alanyl–D-serine in strains with either natural or acquired resistance to vancomycin (22). The VanA-type vancomycin resistance gene cluster in *Streptomyces coelicolor* consists of seven genes in four different operons, *vanRS*, *vanJ*, *vanK*, and *vanHAX*, which 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 (15, 23). The vancomycin resistance gene cluster provides resistance to both vancomycin and teicoplanin and is located on the genome of the vancomycin producer *Amycolatopsis mediterranei* (24, 25) as well as that of other actinomycetes, including the model species *Streptomyces coelicolor* A3 (26, 27).

Streptomycetes are Gram-positive soil bacteria with a complex multicellular life style (28–30). Streptomycetes are a major source of antibiotics and many other natural products of medical and biotechnological importance, such as anticancer, antifungal, or herbicidal compounds (31, 32). Due to the competitive environment of the soil, these microorganisms readily exchange genetic

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material, including antibiotic biosynthetic clusters and antibiotic resistance (33, 34). *S. coelicolor* is a nonpathogenic and genetically tractable model system for vancomycin resistance, with a well-annotated genome (35). The vancomycin resistance cluster of *S. coelicolor* consists of *vanRS*, encoding a two-component regulatory system (TCS) consisting of sensory kinase VanS and response regulator VanR, which 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, and VanX sharing 61%, 63%, and 64% amino acid identity, respectively, while the TCS components VanR and VanS share 31% and 25% amino acid identity, respectively (27, 36). 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* (37). VanH produces D-Lac from pyruvate (38), VanA is a D-alanyl–D-lactate (D-Ala–D-Lac) ligase (39, 40), VanX hydrolyzes the D-Ala–D-Ala dipeptide and has been the target of previous studies assessing vancomycin sensitivity and resistance (36, 41), and VanK attaches glycine to lipid II with D-Lac as the terminal residue (27, 42). VanJ is not required for vancomycin resistance but is instead involved in the resistance to teicoplanin (43). 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 (27, 42, 44, 45). 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 MIC. *Escherichia coli* strains JM109 (46) and ET12567 (47) were used for routine cloning procedures and for extracting nonmethylated DNA, respectively. Cells of *E. coli* were grown in Luria-Bertani broth (LB) at 37°C. *Streptomyces coelicolor* A3 (26) M145 was the parent of all mutants described in this work. All media and routine *Streptomyces* techniques were as described previously (47). Soy flour mannitol (SFM) 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 medium with phosphate (NMMP) supplemented with 1% (wt/vol) mannitol as the sole carbon source. The MICs of vancomycin against *S. coelicolor* M145 and its mutant derivatives were determined by growth on minimal medium (MM) agar plates supplemented with 1% mannitol as the sole carbon source and 0, 2, 4, 8, 16, 32, 64, 128, 256, or 512 μg ml^{-1} vancomycin, in combination with 0, 1, 5, 10, or 50 mM D-Ala or L-Ala. Due to their much higher vancomycin sensitivity, *vanX* mutants were tested with 1, 5, 10, 50, and 100 μM D-Ala and L-Ala.

Five *vanA*-positive *Enterococcus faecium* strains collected in 2011 and 2014 from patients at the Erasmus University Medical Centre, Rotterdam, The Netherlands, were used. The 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 profiles of these isolates (see Table S2 in the supplemental material) were determined using the Vitek II (bioMérieux) 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 the optical density at 600 nm (OD_{600}) reached 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 phenol red; 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 to 24 h at 37°C and MIC values determined visually following the CLSI guidelines or by absorbance at 600 nm.

Constructs for gene disruption and complementation. Deletion mutants were constructed according to a method described previously (48). For deletion of *ddl*, the nucleotide (nt) −948/+20 and +1173/+2638 regions relative to the translational start of *ddl* were amplified by PCR using primer pairs ddl_LF-ddl_LR, and ddl_RF-ddl_RR, using PCR conditions as described previously (49). The left and right flanks were cloned into the multicopy vector pWHM3 (50), which is highly unstable in *Streptomyces* and therefore allows efficient gene disruption (51). 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 nt −1477/+30 and +572/+2035 regions relative to the start of *vanX* (SCO3596) were PCR amplified using primer pairs vanX_LF-vanX_LR and vanX_RF-vanX_RR (see Table S3 in the supplemental material). Insertion of an *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, which expresses the Cre recombinase (52).

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 −573/+1184 relative to the start of *ddl*) (see Table S3 in the supplemental material) and inserted as an EcoRI/BamHI fragment in pHJL401 (53), a highly stable low-copy-number vector that is well suited for genetic complementation (54), 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 previously (55). Equal amounts of unlabeled vancomycin and Vanco-FL were added to the sample to a final concentration of 1 μg/ml, and this was incubated for 10 to 20 min at 30°C. Directly after taking the first sample, 50 mM D-Ala was added to the medium, and the sample was left to grow for another hour before imaging the effect of added D-Ala. Imaging was done as described previously (56). A Zeiss observer with a Plan-Neofluar 40×/0.9 lens was used, and green fluorescent protein (GFP) was excited at a wavelength of 488 nm and observed at 515 nm with filter BP505-550, with the illumination power set to 7.5%. The images were analyzed with ImageJ, and all the fluorescent images were processed identically. The final figure was made with Adobe Photoshop CS6.

Isolation of cytoplasmic PG precursors. For cytoplasmic peptidoglycan (PG) precursor isolation and identification, we used a modification of the method described previously by Hong and colleagues (27). Where applicable, 10 μg vancomycin was added to the strains at the moment of inoculation. The strains were grown in NMMP (1% [wt/vol] mannitol, 50 mM $MgCl_2$) until mid-log phase (OD of 0.3 to 0.4), and mycelia were harvested by centrifugation at 4°C and washed in 0.9% NaCl. Mycelia were extracted with 5% cold trichloroacetic acid (TCA) for 30 min at 4°C. This product was centrifuged and the supernatant desalted on a Sephadex G-25 column (Illustra NAP-10 columns; GE Healthcare, Pittsburgh, PA) and concentrated by rotary evaporation. The concentrated precursors

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TABLE 1 Effect of D-Ala on the MICs of vancomycin against *S. coelicolor* M145 and its mutant LAG2

Strain	Vancomycin MIC (μg/ml) with:						
		D-Ala			L-Ala		
	No amino acid	5 mM	10 mM	50 mM	5 mM	10 mM	50 mM
M145	128	32	32	4	128	128	128
Δ*ddl* mutant[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 had an MIC identical to that of M145.

were dissolved in high-pressure liquid chromatography (HPLC)-grade water and separated by liquid chromatography-mass spectrometry (LC-MS) using a gradient of 0 to 20% acetonitrile in water with 0.1% trifluoroacetic acid (TFA). The elution was monitored at 254 nm and by the sizes eluted (*m/z* 1193.8 to 1195.3).

For the measurement over time, the protocol was adjusted in the following way. NMMP cultures (300 ml) were grown until exponential phase (OD of 0.3 to 0.4), at which point a 10-ml sample was taken ($t = 0$) and 50 mM D-Ala or L-Ala was added to the original culture, followed by further sampling after 1, 5, 15, 30, 60, 120, and 180 min. Samples were rapidly filtered with a vacuum pump and washed with 0.9% (wt/vol) NaCl, and mycelia were 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 imply that it can use both D-Lac and D-Ala as substrates (40, 57), 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 medium (MM) agar plates with increasing concentrations of D-Ala and vancomycin. D-Ala was added at a concentration 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 medium with up to 50 mM L-Ala did not have any effect on the susceptibility to vancomycin (Table 1; see Fig. S1 in the supplemental material). 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; see Fig. S1 in the supplemental material). 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 (58, 59).

Creation of a vancomycin-independent *ddl* mutant. To study the molecular basis of this effect in more detail, a strain that depends on *vanA* for the synthesis of the D-Ala–D-Ala dipeptide and thus for cell wall synthesis was required. The wild-type gene for D-Ala–D-Ala ligase is *ddl* (SCO5560 in *S. coelicolor*), which is essential for normal growth, but its absence can be rescued by the vancomycin-inducible expression of *vanA*, the only other paralogue of *ddl* in the *S. coelicolor* genome (40, 60). To allow direct comparison with other mutants related to GlcNAc and cell wall metabolism previously made in our laboratory (61–63), 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). 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 (42). 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 shown). As expected, the *ddl* mutant could grow only 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 shown).

To allow study of 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

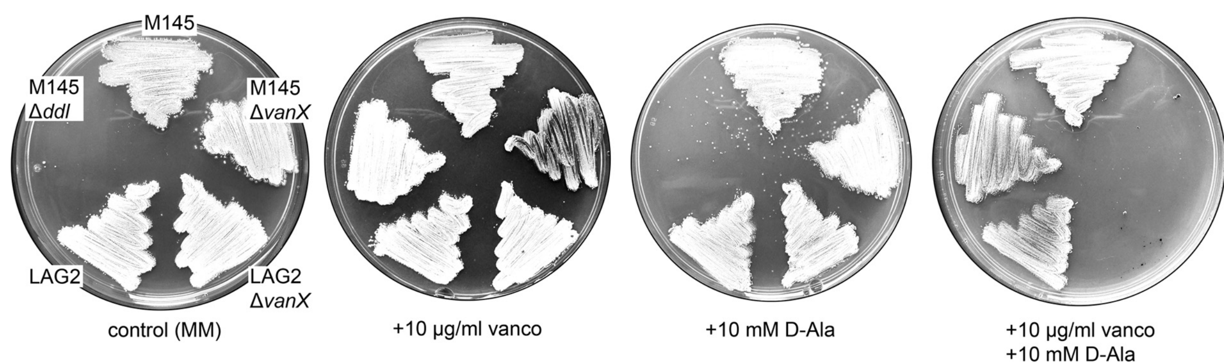

FIG 1 Effect of D-Ala on growth of *S. coelicolor* M145 and derivatives. The strains are *S. coelicolor* M145 (parental strain), its *vanX* mutant, suppressor mutant LAG2, LAG2 Δ*vanX* and M145 Δ*ddl*. Strains were streaked on MM with, from left to right, no additives (control), 10 μg/ml vancomycin, 10 mM D-Ala, or 10 μg/ml vancomycin plus 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.

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TABLE 2 MICs of vancomycin against *S. coelicolor vanX* mutants with D-Ala

Strain	Vancomycin MIC (μg/ml) with:						
		D-Ala			L-Ala		
	No amino acid	10 μM	50 μm	100 μM	10 μM	50 μM	100 μM
M145 Δ*vanX*	32	16	1	1	32	32	32
LAG2 Δ*vanX*	64	32	8	2	32	32	32

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}. 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).

DNA sequencing of the vancomycin resistance genes *vanRSJKHAX* of strain LAG2 revealed that the insertion element IS*466A* (SCO3469) (16, 64, 65) had inserted at nt 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 *Enterococcus* strains and results in constitutive upregulation of the vancomycin resistance cluster (37, 66, 67). The *ddl* suppressor mutant LAG2 had a level of vancomycin resistance similar to that of the parental strain, with an MIC of 128 μg/ml (Table 1). Similar to what is 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 vancomycin MIC comparable to that for 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 (68, 69). A *vanX*-null mutant was created using a strategy similar to that for *ddl*, replacing the coding region of *vanX* by the apramycin resistance cassette *aacC4*. The mutant was created from both the parental strain *S. coelicolor* M145 and 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 10 μg/ml vancomycin and 10 mM D-alanine but failed to grow on medium containing both vancomycin and D-alanine at a concentration where M145 and LAG2 did not show sensitivity to vancomycin (Fig. 1). LAG2 Δ*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 LAG2 Δ*vanX*, 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 4,000 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 compared to the *vanX*-positive parental strain.

Analysis of PG precursors. In order to get more insight into the synthesis of vancomycin-sensitive (i.e., wild-type) or vancomycin-resistant peptidoglycan (PG), the pool of PG precursors was analyzed by liquid chromatography coupled to mass spectrometry (LC-MS) (58, 60, 70) 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 1,994 Da and a retention time of around 7.2 min, while vancomycin-insensitive precursors ending with D-Ala–D-Lac are characterized by a peak of a monoisotopic mass of 1,995 Da and a significantly higher retention time of around 8.2 min (Fig. 2A).

In extracts from the parental strain grown in the absence of vancomycin, only wild-type precursors were observed (Fig. 2B). As expected, when *S. coelicolor* M145 was grown in the presence of 10 μg/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. 2B). 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. 2B). 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 and not by 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 a 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 at 3 h after the addition of D-Ala (Fig. 2). This supports the notion that in the absence of VanX, wild-type precursors are incorporated into the cell wall much more frequently, with increased sensitivity to vancomycin as a consequence.

Visualization of vancomycin binding by fluorescence microscopy. To qualitatively determine the ability of vancomycin to

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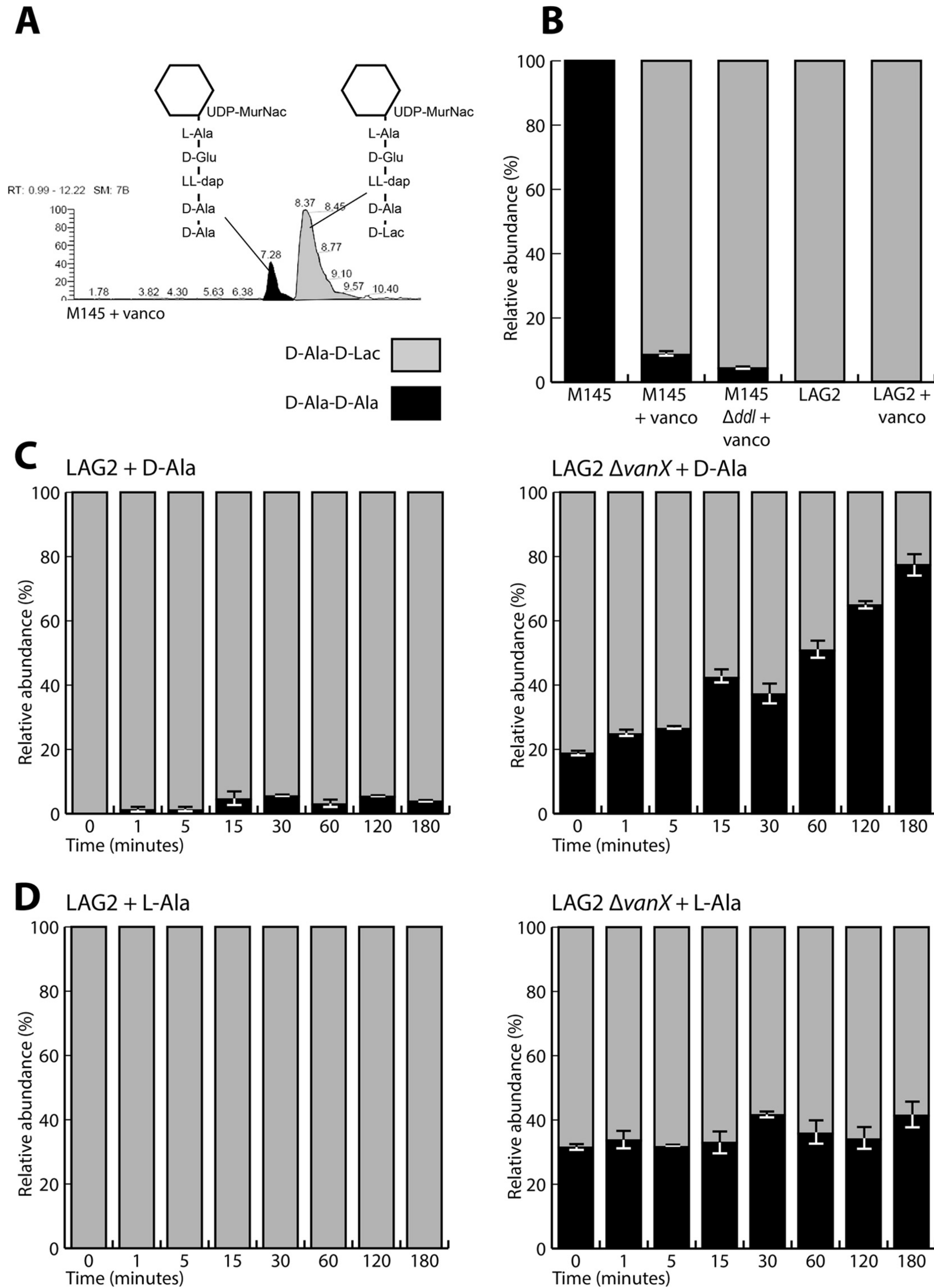

FIG 2 LC-MS analysis of peptidoglycan precursors. (A) Example peak profile and corresponding precursors of *S. coelicolor* M145 grown with vancomycin, with the peak area corresponding to a precursor terminating in D-Ala–D-Ala shown in black and the peak corresponding to a precursor terminating in D-Ala–D-Lac in gray. (B) Ratio (%) of wild-type (black) and vancomycin-resistant (gray) 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 shown only with vancomycin, as it fails to grow in its absence. LAG2 with and without vancomycin has less than 1% wild-type (vancomycin-sensitive) peptidoglycan. Strains were grown with or without vancomycin to an OD of 0.3 to 0.4 before harvesting. (C) Accumulation of wild-type and vancomycin-resistant precursors over time in LAG2 and LAG2 Δ*vanX*. The samples were grown to an OD of 0.3 to 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 min after the addition of D-Ala. (D) Same as for panel C but with L-Ala instead of D-Ala. Bars representing the precursors are shown as percentages (with the total set to 100%).

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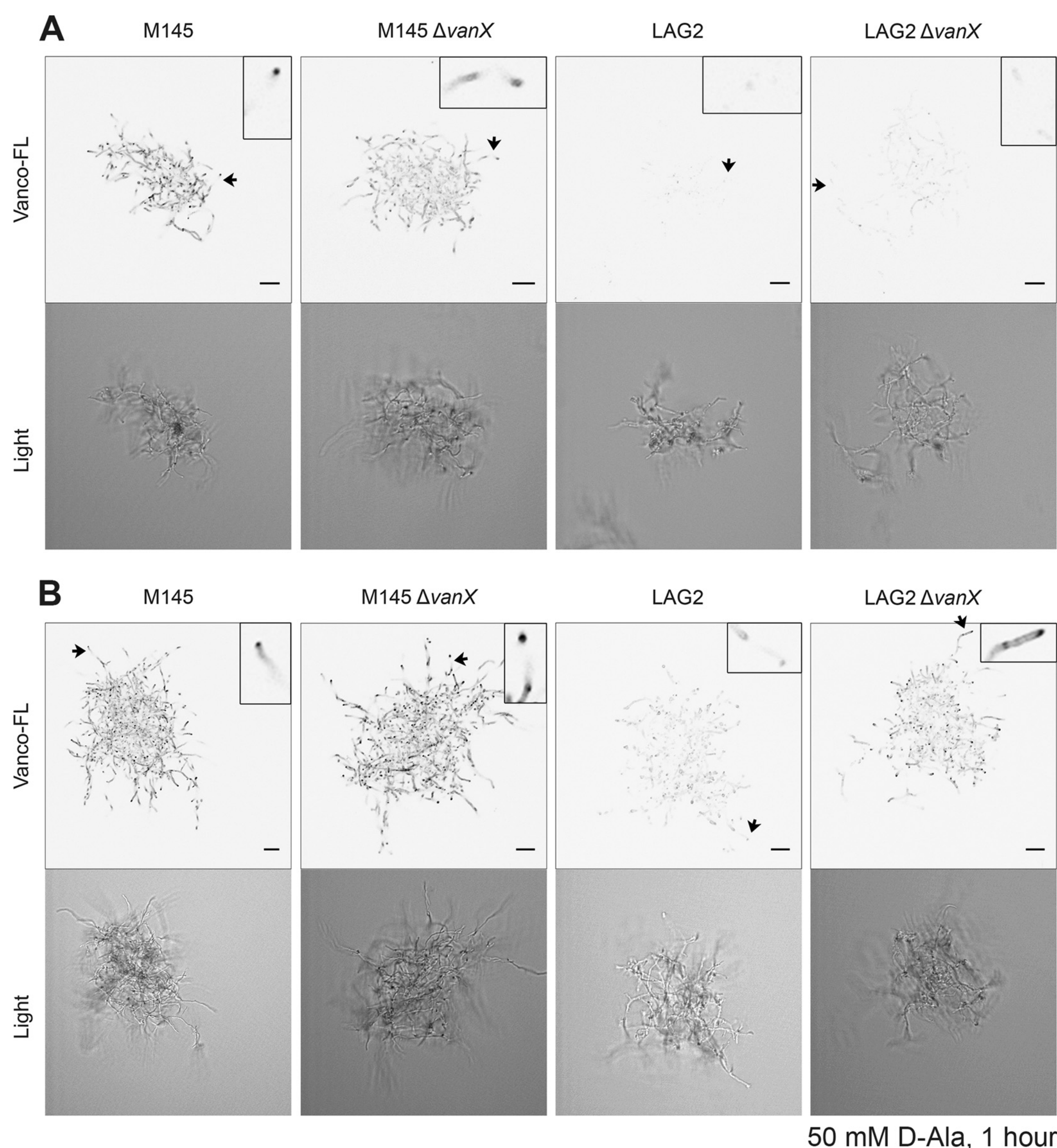

FIG 3 Fluorescence micrographs of Vanco-FL-stained hyphae. To analyze vancomycin binding, *S. coelicolor* strains M145, M145 Δ*vanX*, LAG2, and LAG2 Δ*vanX* were grown in liquid NMMP for 12 h and continued to grow for 1 h in the absence (A) or presence (B) of 50 mM D-Ala. Mycelia were then stained with Vanco-FL and imaged. Top panels, fluorescence micrographs (inverted grey scale); bottom panels, 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 LAG2 Δ*vanX* only after the addition of D-Ala. Insets show magnifications of the areas indicated by arrows in the respective images. Scale bars, 10 μm.

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 (55). In *S. coelicolor*, which grows by tip extension (71), 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 LAG2 Δ*vanX*) (Fig. 3A). However, addition of D-Ala resulted in marginal staining by Vanco-FL of the LAG2 hyphal tips (Fig. 3B); in contrast, its *vanX* mutant derivative LAG4 was stained very well, in line with the strongly enhanced vancomycin sensitivity of the mutant (Fig. 3).

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).

Analysis of the effect of D-Ala on the MICs 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 iso-

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TABLE 3 MICs of vancomycin against VRE in the presence or absence of D-Ala

Strain	Vancomycin MIC (μg/ml) with D-Ala at:		Dilution step reduction
	0 mM	50 mM	
vanA1	4,096	256	4
vanA2	4,096	256	4
vanA3	4,096	128	5
vanA4	4,096	128	5
vanA10	2,048	16	7

lates of *E. faecium*. MIC values were calculated by testing a serial (2-fold) dilution of vancomycin in the presence or absence of D-Ala in triplicate (Table 3). Similar to what was seen for *S. coelicolor*, addition of 50 mM D-Ala to the growth medium resulted in a strong increase in the efficacy of vancomycin against all clinical isolates, with reduction of 4 to 7 dilution steps. Even in the worst cases, the MIC of vancomycin was still reduced 16- to 32-fold (from 4,096 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.

DISCUSSION

With the rapid spread of vancomycin resistance, new efforts are needed to maintain this last-resort antibiotic as a clinical drug against multidrug-resistant bacterial infectious diseases. So far, attempts have included engineering VanX inhibitors (26, 41, 72) or reengineering 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 (73). As a basis to develop new approaches to target vancomycin resistance, we studied the model organism *S. coelicolor*, which has a set of vancomycin resistance genes very similar to those of the pathogenic VRE (34).

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, with the affinity for either D-Lac or D-Ala as a substrate being highly dependent on the substrate and pH (45, 74, 75). The extracellular addition of high concentrations of 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 (58, 60, 70). 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. We have also tested whether the effect of D-Ala was apparent for A40926, the natural precursor of the expanded-spectrum semisynthetic glycopeptide antibiotic dalbavancin isolated from *Nonomuraea* sp. strain ATCC 39727 (76). Perhaps surprisingly, the efficacy of A40296 was not affected by the addition of D-Ala (data not shown). This suggests that its mode of action and the mechanism of resistance are different from those of vancomycin, despite the fact that heterologous expression of the *vanHAX* cluster increases resistance to A40926 in *Nonomuraea* spp. (77).

Importantly, the effect of D-Ala as enhancer of the efficacy of vancomycin was 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, which is 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 DD-transpeptidases in the periplasm substituting D-Lac for D-Ala on the precursors or by inhibition of the expression of D-Lac dehydrogenases by D-Ala. The strong direct correlation between PG precursor accumulation and vancomycin binding (as determined by imaging fluorescent vancomycin) argues against a major influence of DD-transpeptidases in this process. 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 not only that D-Ala is incorporated in PG precursors but that the pentapeptides terminating in D-Ala–D-Ala are displayed at the cell surface and incorporated into the mature cell wall. 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 (70). However, as our work shows, significant changes in the MIC are brought about only 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. 4). In the presence of excess 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 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 a 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 to 50 μM instead of 10 to 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 μg/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 such as VRE and vancomycin-resistant *S. aureus* (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,

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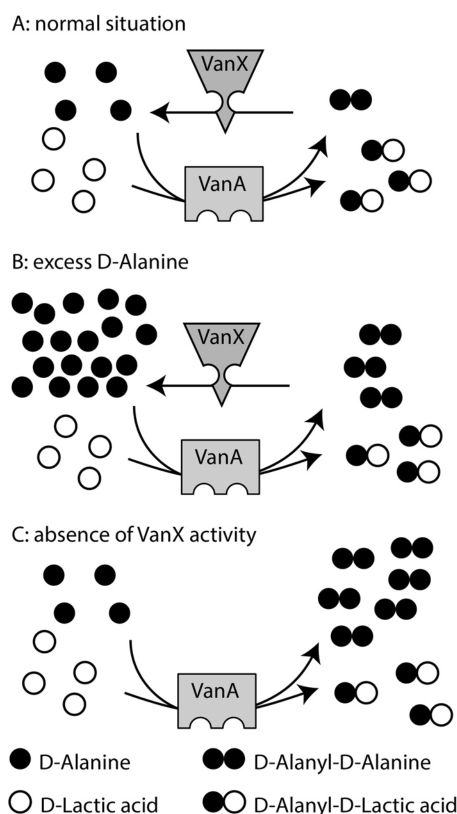

FIG 4 Model of how D-Ala influences the activity of VanA in the presence or absence of VanX. All drawings show the situation where VanA is constitutively expressed and in the absence of Ddl. (A) Normal situation. VanA produces both D-Ala–D-Ala and D-Ala–D-Lac, whereby D-Ala–D-Ala is broken down by VanX (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 a 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 excess D-Ala is added. This then enhances the percentage of wild-type cell wall precursors and strongly amplifies the efficacy of vancomycin.

but their effect was limited (26, 41, 72, 78, 79). 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, decreasing the MIC by only 2-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 are candidates as screening hosts for a high-throughput screen of small molecules that target vancomycin resistance. This may prove to be an important asset in the hunt for drugs that counteract vancomycin-resistant pathogens such as VRE and VRSA.

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L.T.V.D.A. contributed to the conception and design of the study, performed the work on *S. coelicolor*, and wrote and revised the article. N.L. performed the work on *E. faecium*. W.J.V.W. designed the work on *E. faecium* and wrote the article. G.P.V.W. contributed to the conception and design of the study and wrote and revised the article. All authors read and agreed on the final version of the article.

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