Characterisation of the effector ligand recognised by VanS, a key protein triggering resistance to vancomycin

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Introduction. The glycopeptide antibiotic vancomycin is the front-line therapy for treating hospital acquired infections caused by some pathogenic bacteria such as Enterococci and Staphylococci. Vancomycin inhibits bacterial cell wall biosynthesis by binding to the D-alanyl-D-alanine (D-Ala-D-Ala) terminus of lipid-attached peptidoglycan (PG) precursors, blocking formation of the mature PG that gives the cell wall its rigidity [1]. The spread of resistance to glycopeptide antibiotics through bacterial populations is an acute public health issue, as highlighted by the emergence of vancomycin-resistant MRSA (VRSA) in hospitals [2]. The first clinical isolates of vancomycin-resistant infections emerged in Enterococcal strains in the mid-1980's, and was shown to result from the replacement of the D-Ala-D-Ala dipeptide terminus of cell wall PG precursors by D-alanyl-D-lactate (D-Ala-D-Lac) which reduces the affinity of vancomycin binding by 1000-fold. This alteration in cell wall biosynthesis requires the expression of at least three genes, *vanHAX*, which is usually dependent on a VanRS two-component regulatory system [3]. One of the most important questions yet to be answered in the study of vancomycin resistance is the nature of the specific ligand recognised by the VanS sensor protein. Two distinct models have been proposed: i) direct induction, in which the sensor kinase is activated by direct binding of antibiotic to the sensor domain; ii) indirect induction, in which the sensor kinase is activated by binding a cell wall metabolite that is either intermediate in cell wall biosynthesis or accumulates as a result of antibiotic action. These models are however not mutually exclusive, since an intermediate possibility exists whereby VanS is induced by the glycopeptide antibiotic when it is bound to a D-Ala-D-Ala-containing cell wall metabolite. A significant amount of effort has been put into studying the different VanR/VanS two-component systems in clinical isolates of enterococcal strains but the exact nature of the direct molecular ligand that activates VanS has remained elusive.

Methods. In this study, we will present some result that we gained through working on the the *van* cluster in *Streptomyces coelicolor* as a model for the VanB resistance system. We have undertaken a series of *in vivo* studies including bioassays using glycopeptide-dependent mutant strain and assays using a semi-synthetised vancomycin photoaffinity probe (VPP). We have also attempted to manipulate the relative amounts of D-Ala-D-Ala and D-Ala-D-Lac containing cell wall PG precursors in *S. coelicolor* cell genetically. Complementation of an essential D-Ala-D-Ala ligase activity by constitutive expression of vanA encoding a bifunctional D-Ala-D-Ala and D-Ala-D-Lac ligase activity allowed construction of strains which synthesised variable amounts of PG precursors containing D-Ala-D-Ala. Assays quantifying the expression of genes under VanRS control to prove that the response to vancomycin in these strains correlated with the abundance of D-Ala-D-Ala-containing PG precursors were also carried out. .

Results. A range of antibiotics were tested for their ability to induce the VanR/VanS system in *S. coelicolor* using Δ*femX* bioassay systems [4] and the inducers identified are all structurally closely related glycopeptide antibiotics, suggesting that VanS interacts directly with glycopeptide antibiotics. In addition, vancomycin photoaffinity probe directly binds to VanS and that this binding is required for the expression of the *van* genes and for resistance to vancomycin [5]. These results strongly favour the direct induction mechanism over the indirect one thus the specific ligand directly perceived by VanS must be vancomycin itself. In addition, we also observed that strains producing a lower proportion of PG precursors terminating in D-Ala-D-Ala consistently exhibited a lower response to vancomycin (Fig. 1). Pre-treatment of wild-type cells with vancomycin or teicoplanin to saturate and mask the D-Ala-D-Ala binding sites in nascent PG also blocked the transcriptional response to

subsequent vancomycin exposure (Fig. 2), and desleucyl vancomycin, a vancomycin analogue incapable of interacting with D-Ala-D-Ala residues, failed to induce *van* gene expression (Fig. 3) [6].

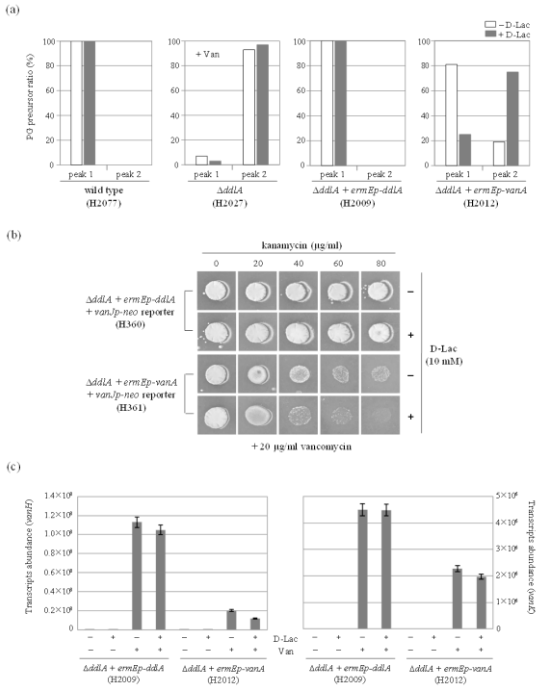

Fig.1 Induction of *van* gene expression by vancomycin is significantly reduced in cells where D-Ala-D-Lac containing PG precursors are more abundant than D-Ala-D-Ala containing PG precursors. (a) Relative abundance of the D-Ala-D-Ala-containing PG precursor (UDP-MurNAc-pentapeptide) and the D-Ala-D-Lac-containing PG precursor (UDP-MurNAc-pentadepsipeptide) in wild type (H2077), Δ*ddlA* (H2027) and the Δ*ddlA* mutant complemented with *ermEp-ddlA* (H2009) or *ermEp-vanA* (H2012) when grown in the presence (grey bar) or absence (white bar) of a 10 mM D-Lac supplement. Cells grown to mid-late exponential phase in NMMP liquid cultures were extracted and analysed by HPLC-MS/MS. Relative abundances were calculated by normalising each sample so that the sum of the two UDP-MurNAC derivatives equaled 100. Strain H2027 (Δ*ddlA*) is viable only in the presence of vancomycin and was therefore grown in NMMP containing 20 μg/ml vancomycin as indicated by +Van. (b) Bioassay for kanamycin resistance induced from the vancomycin inducible *vanJ* promoter (*vanJp-neo*) in the reporter strains. Approximately 105 spores were spotted onto agar plates containing 20 μg/ml vancomycin, kanamycin at a range of concentrations between 0 and 80 μg/ml in 20 μg/ml increments, and in the presence (+) or absence (-) of 10 mM D-Lac. The result was scored after 4 days of incubation at 30°C. (c) Induction of *vanH* and *vanK* transcription in Δ*ddlA* mutant complemented with *ermEp-ddlA* (H2009) and *ermEp-vanA* (H2012) in response to vancomycin, as determined by qRT-PCR. Each strain was grown to mid-late exponential phase in two different NMMP liquid media, one with 10 mM D-Lac (+) and one without (-). Samples were then taken immediately before the addition of 10 μg/ml vancomycin (-) and 30 min after treatment (+). Total RNAs were extracted from each sample and qRT-PCR was carried out.

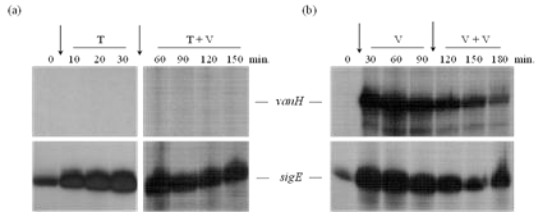

Fig.2 Saturation of the D-Ala-D-Ala termini of immature PG by pretreatment of cells with (a) teicoplanin or (b) vancomycin prevents the sensing of vancomycin by VanS. Please note that the glycopeptide antibiotic teicoplanin is structurally very similar to vancomycin and also binds to the D-Ala-D-Ala termini of extracellular PG precursors. In contrast to vancomycin however, it does not inhibit the binding of a vancomycin photoaffinity probe to VanS in vitro, nor does it act as a positive inducer of VanS activity in bioassays. Wild type *S. coelicolor* M600 cells were grown to mid-late exponential phase in NMMP liquid medium and pretreated at time 0 by addition of 50 μg/ml teicoplanin (a) or 10 μg/ml vancomycin (b), as indicated by the vertical arrows. Samples were taken at the intervals shown before addition of a further 10 μg/ml vancomycin (arrowed) after 30 min (a) or 90 min (b). RNA was extracted from each sample and *vanH* transcript abundance determined by S1 nuclease protection. Transcription of *sigE* is shown as a positive control for cell viability and RNA integrity, and as an indicator of cell wall stress. Where shown, V represents vancomycin and T indicates teicoplanin.

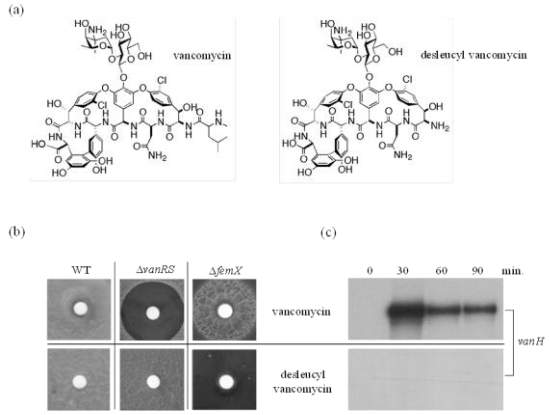

Fig.3 Desleucyl vancomycin which does not bind to the D-Ala-D-Ala termini of extracellular PG precursors fails to induce *van* gene expression. (a) Structure of vancomycin and desleucyl vancomycin. (b) Bioassay analysing vancomycin and desleucyl vancomycin activity against *S. coelicolor* wild type M600 (vancomycin resistant), Δ*vanRS* mutant (vancomycin sensitive) and Δ*femX* (vancomycin dependent) strains. Paper discs containing 30 μg of each drug were placed on freshly spread lawns of each strain and plates were incubated for 4 days at 30°C. The desleucyl derivative failed to stimulate the halo of growth of the Δ*femX* strain that was clearly evident around the vancomycin disc. In addition, the large halo of growth inhibition produced by exposure of the Δ*vanRS* strain to a disc containing vancomycin was completely absent when a desleucyl vancomycin disc was used. (c) Response of the *vanH* promoter to vancomycin or desleucyl vancomycin in *S. coelicolor* M600. Cells were grown to mid-late exponential phase in NMMP liquid medium and exposed to 10 μg/ml vancomycin or 10 μg/ml desleucyl vancomycin. Total RNAs were extracted from samples taken immediately before the addition of

drug and at subsequent 30 min intervals and analysed by S1 nuclease protection assays.

Conclusions. As a consequence, we claim that vancomycin is the direct ligand for VanS but vancomycin primarily requires binding to the D-Ala-D-Ala termini of cell wall PG precursors to be perceived by the VanS sensor protein. The instant impact of the outcome of the study regarding the specific ligand for VanS may enable us to design new glycopeptide antibiotics, consequently outsmarting glycopeptide resistance in clinical infection (Fig. 4).

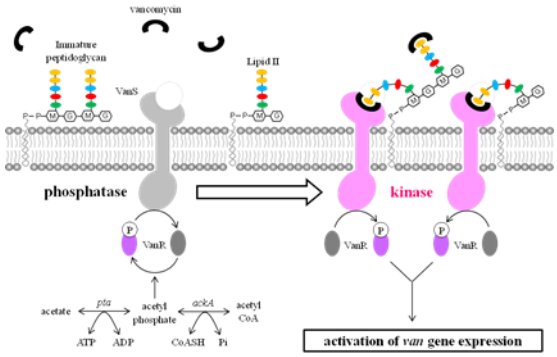

Fig.4 A model proposing that induction of the vancomycin resistance system in *S. coelicolor* via VanS kinase activity requires binding of the drug to D-Ala-D-Ala termini of the extracellular cell wall PG precursors. Vancomycin (black cup shapes) bound to D-Ala-D-Ala termini of extracellular PG precursor is perceived by VanS which switches its activity from phosphatase to kinase causing the accumulation of phospho-VanR which in turn induces the expression of the *van* gene cluster and renders the cell resistant to vancomycin. Unbound vancomycin is not perceived by the VanS sensor and VanS retains the phosphatase activity of its non-induced state.

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