new horizons in CF-research

Swiss Working Group for Cystic Fibrosis

Report of a research project

Project Title:	Exploring lysis and adaptive resistance to vancomycin in (leaky) *Pseudomonas aeruginosa*
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Background

Children, adolescent and adults suffering from the genetic lung disease cystic fibrosis (CF) are especially prone to acquire infections of the Gram-negative (diderm) pathogen *Pseudomonas aeruginosa*. Aggressive antibiotic treatment regimes, including the cell wall-targeting beta-lactam (β-lactam) antibiotics, are currently employed successfully, increasing the life expectancy of *P. aeruginosa*-infected patient with CF. Unfortunately, many of these patients eventually succumb to the infection because of the difficulty of eradicating *P. aeruginosa* (*PA*) completely, owing to a myriad of natural or acquired antibiotic tolerance and/or resistance mechanisms protecting *P. aeruginosa*[1-2].

In diderm, the bacterial the cell wall (also known as peptidoglycan, PG) is an envelope layer that is sandwiched in between the cytoplasmic membrane (CM) and the outer membrane (OM). The PG is an ideal drug target because this structure and the PG building block are not present in human cells and because it is required to protects cells from lysis induced by internal osmotic pressure and/or other mechanisms. β-lactam antibiotics are extremely efficient drugs that perturb PG synthesis and promote cell lysis, but there are numerous clinical PA isolates which have become resistant to β-lactams and are thus difficult to cure. Resistance mechanisms include 1) β-lactam-induced activation of an enzyme (β-lactamase, AmpC) that destroys the drug, 2) active expulsion of the drug from the cell by efflux pumps to avoid its action on the PG, and/or 3) reduced passive permeability of β-lactams across the outer membrane (OM) to reach the PG target (for example by mutations lessening the OprD porin expression to reduce drug passage across the OM)[3]. Alternative approaches, potentially combination therapies, must be considered with PG-targeting antibiotics to treat *PA* infections.

Because of these resistance mechanism to β-lactams in *PA*, other PG targeting may provide an alternative route to therapy. However, many PG-targeting antibiotics such as the natural glycopeptide vancomycin (VNC) are often less effective against Gram-negative bacteria because the OM obstructs passage of VNC to reach its target. Nonetheless, VNC may be potentially be used in combination with other molecules that permeabilize the OM and this render *PA* susceptible to VNC. Here we examined the transcriptional response of permeable *PA* (genetically engineered to have a leaky OM) to VNC.

Results and Conclusions

Part1: Cell-wall targeting antibiotics profiling of WT and leaky mutants of PA14

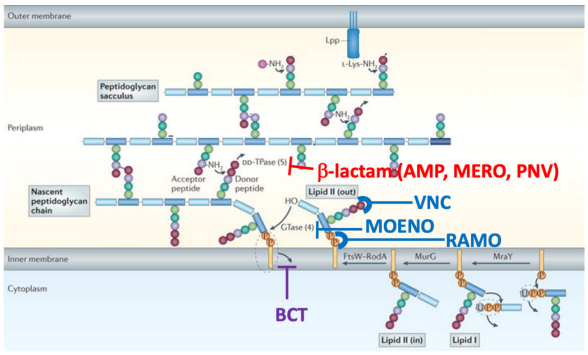
We previously determined that a PA14 strain harboring an hypomorphic allele of the LPS transporter LptD (*lptD4213*) allele is at least 30 times more sensitive to VNC than the wild-type (*WT*) strain (Figure 1A) because its OM becomes permeable to VNC (leaky phenotype), allowing the antibiotic to reach its target in the periplasm. We have extended this analysis to other antibiotics targeting PG synthesis as well as to another mutant with a leaky OM that we have recently identified. This strain harbors a deletion in *bamB* which codes for a non-essential lipoprotein of the BAM complex, a machinery that inserts β-barrel protein in OM. While the *lptD4213* mutant was much more sensitive than the wild-type to several PG-targeting antibiotics, but also β-lactams, the Δ*bamB* show a higher sensitivity to antibiotics targeting the PG precursor lipid II (Figure 1A and 1B). Further work showed that the deletion of 40 identifiable OM porins (OMP) encoded in the PA14 genome[4] reduced the VNC sensitivity of Δ*bamB* mutant, but not the *lptD4213* strain (Figure 1C). Thus, we conclude that the OM permeability of these two strains is caused by different alterations to the OM

structure. A large body of work in *E. coli* has shown that the *lptD4213* mutation reduces the amount of LPS in outer leaflet of the OM rendering it much more permeable to various chemicals[5].

In conclusion, based on the result we obtained here and on previous study from our laboratory showing that an OM TonB-dependent receptor (TBDR) mediates the VNC sensitivity of *Caulobacter crescentus*, a Gram-negative bacteria that is unusually sensitive to this antibiotic[6], we hypothesize that VNC and other lipid II targeting antibiotics could cross the OM of the Δ*bamB* mutant through one or several OMPs or through a stalled OMP-BAM complex. While other explanations are also possible, we are investigating the precise mechanism of this VNC sensitivity.

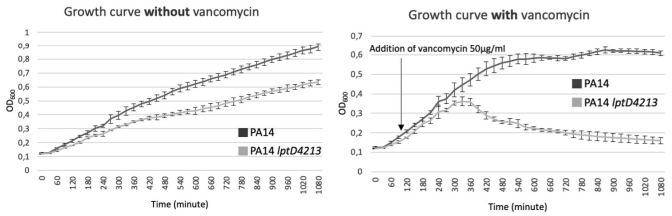
A

		MIC		
		PA14	*lptD4213*	*ΔbamB*
β-lactam	Ampicillin (AMP)	512	16	256
	Meropenem (MERO)	1,00	0,25	1,00
	Penicillin V (PNV)	>8000	125	2000
	Vancomycin (VNC)	>1024	32	64
	Moenomycin (MOENO)	>16	2	8
	Ramoplanin (RAM)	>512	32-64	64
	Bacitracin (BCT)	>2048	128-256	1024



Adapted from *Typas, A., Banzhaf, M., Gross, C.A. & Vollmer, W. From the regulation of peptidoglycan synthesis to bacterial growth and morphology. Nat Rev Microbiol 10, 123-136 (2011).*

B



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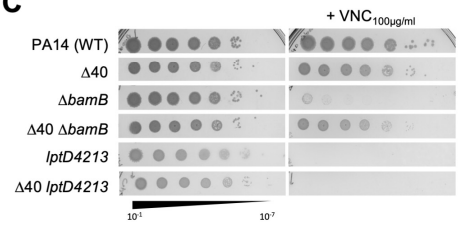


Figure 1 Two leaky, vancomycin-sensitive strains of *P. aeruginosa* PA14 have different sensitivity profiles to peptidoglycan-targeting antibiotics and potential vancomycin entry pathways through the outer membrane. A) Left panel, minimum inhibitory concentration (MIC in μg/ml) of the indicated cell-wall targeting antibiotics for wild-type (PA14), *lptD4213*, and Δ*bamB* mutants. Right panel, scheme showing the steps of peptidoglycan cell-wall synthesis inhibited by the antibiotics used for the MIC determination. B) Growth curve of wild-type (PA14) and *lptD4213* mutant with and without 50 μg/ml of VNC measured by optical density (OD600). C) Deletion of the genes encoding 40 all identifiable outer membrane porins of PA14 suppresses the vancomycin sensitivity of the Δ*bamB* mutant but not of the *lptD4213* mutant. Efficiency of plating assay of *P. aeruginosa* PA14 WT, porin-less PA14 mutant (Δ40), Δ*bamB*, Δ*bamB* Δ40, *lptD4213*, and *lptD4213* Δ40 strains in absence (CTR) or presence of 100 μg/ml VNC.

Part2: Transcriptional response of WT and leaky lptD4213 mutant of PA14 to VNC

WT PA is extremely resistant to VNC and its *in vitro* growth is only marginally affected by a VNC concentration that leads to effective lysis of the *lptD4213* leaky mutant (Figure 1B) and which is in the higher range of the peak concentrations achieved in infant patients treated for infection with Gram+ (monoderm) bacteria that lack an OM[7]. Surprisingly, transcriptome

analyses by RNA-seq revealed that *WT PA* rapidly responded to VNC exposure by extensively changing its gene expression pattern with more than 500 genes significantly up or down regulated (Figure 2). The *lptD4213* mutant altered almost 2000 transcripts in response to VNC, which is expected as cells were harvested at the onset of cell lysis during VNC treatment. Gene set enrichment analysis showed that VNC affected several gene classes in the *lptD4213* cells, including genes for PG synthesis and OM lipoprotein transport that were upregulated. By contrast, transcripts encoding OMPs and TBDRs were downregulated. Interestingly, VNC exposure also altered 561 transcripts in *WT* cells, of which 200 were not induced in *lptD4213* cells. The latter include transcripts coding for components of the anaerobic respiratory chain (denitrification pathway) which are strongly upregulated (Figure 2, right panel), while transcripts encoding the cytochrome O terminal oxidase complex, a component of the aerobic respiratory chain, are downregulated after VNC treatment. Finally, 300 transcripts are regulated both in WT and in *lptD4213* cells. This includes: the upregulation of the genes implicated in L-Ara4N modification of the lipid A part of the LPS (Figure 2, right panel) and in alginate synthesis, as well as down regulation of the flagellum assembly pathway (Figure 4A), of genes related to pyoverdine synthesis and iron homeostasis, and of genes implicated in dipeptide transport across the OM and the CM.

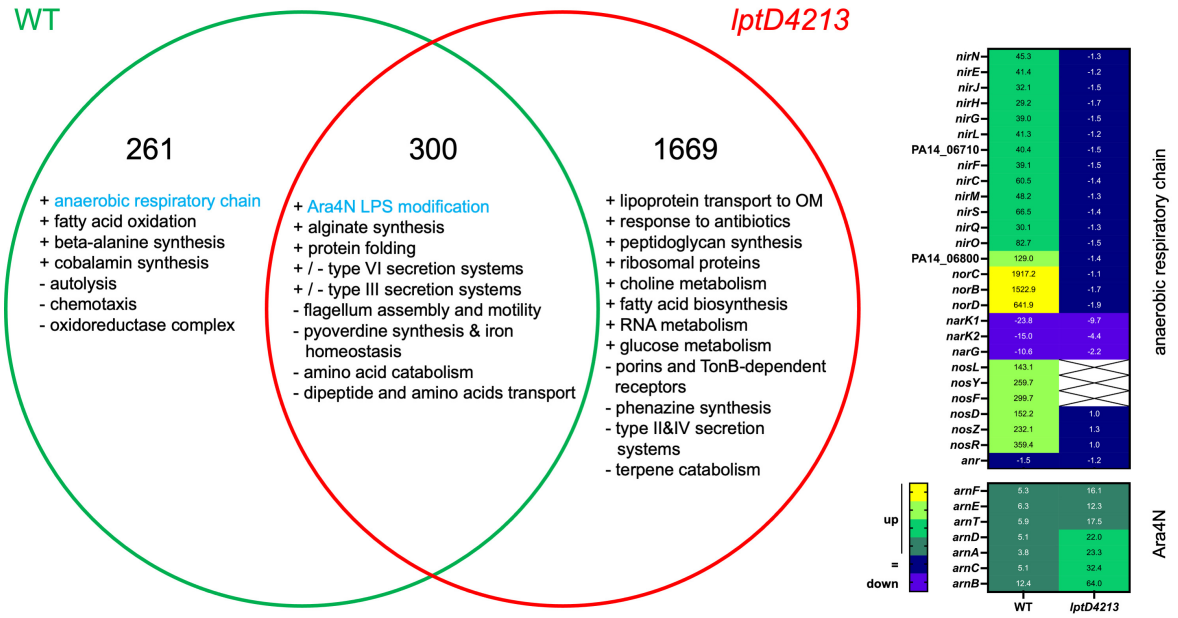
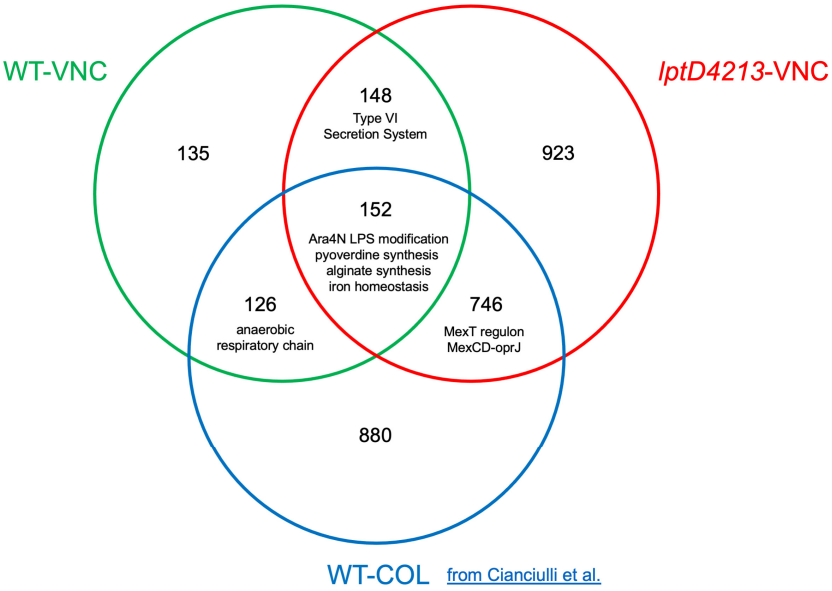


Figure 2 Genome-wide analysis of *P. aeruginosa* PA14 transcriptional response following exposure to vancomycin (VNC) shows that VNC is detected by *WT* cells, despite being VNC-resistant due to the OM. Left panel, Venn diagram showing number of genes significantly up- (>2 fold) or down- (<2 fold) regulated following exposure to VNC (VNC 200 μg/ml, 90 min) in liquid culture at 37°C compared to untreated control in PA14 (*WT*) and *lptD4213* mutant according to RNA-seq data. Genes in each part of the Venn diagram were analyzed by gene set enrichment analysis performed on fold-change value of all genes with at least 50 RNA-seq reads in control or VNC-treated samples using Gene Ontology (GO) categories. Most specific subcategories with significantly up- (+), down- (–), or up and down- (+ / –) regulated gene expression are listed. Right panel, heat map with gene expression fold change upon VNC exposure for PA14 genes included in the beta-L-Ara4N-lipid A biosynthetic process term (GO:1901760) and in anaerobic respiration chain (as listed in [8]).

To better understand which changes are specifically related to VNC exposure and which are linked to a more general envelope stress response caused by the antibiotic, we took advantage of a recently published RNA-seq data set of PA14 in response to inhibitory concentration of colistin (COL), a polycationic cyclic antimicrobial peptide used as a last-resort treatment for multidrug-resistant Gram-negative infections[8]. By comparing genes significantly regulated in PA14 upon exposure to VNC (WT and *lptD4213* mutant) and COL (WT) and performing gene set overrepresentation analysis, we observed 152 transcripts to be affected in the three conditions, with an overrepresentation of genes involved in L-Ara4N modification of LPS, pyoverdine and alginate synthesis, and iron homeostasis, thus potentially defining a response to antibiotic targeting the PA14 cell envelope (Figure 3).



from Cianciulli et al.

Figure 3 Comparison of *P. aeruginosa* PA14 transcriptional response following vancomycin and colistin exposure. Venn diagram showing number of genes significantly up- (>2 fold) or down- (<2 fold) regulated following exposure to vancomycin (VNC 200 μg/ml, 90 min) or colistin (COL 8 μg/ml, 120 min) in liquid culture at 37°C compared to untreated control in PA14 (WT) and *lptD4213* mutant. Genes in each intersection of the Venn diagram were analyzed by gene set overrepresentation analysis (i.e. which does not require numerical values thus allowing for the comparison of data coming from different experiments) using Gene Ontology (GO) categories. Most specific subcategories with significantly overrepresented genes are listed. RNA-seq data for colistin were retrieved from Cianciulli Sesso et al[8].

We also observed that flagellar transcripts are downregulated after exposure to VNC but not by COL (Figure 3), with the downregulation being stronger in *lptD4213* cells versus *WT* cells. To determine whether this reduction in transcript levels is due to a reduction of transcript synthesis, we performed ChIP-seq (chromatin immunoprecipitations followed by deep sequencing) analyses with monoclonal antibodies to various RNA polymerase (RNAP) subunits of *E. coli* that were used to precipitate RNAP from *PA* cells before or after VNC treatment. RNAP ChIP-Seq allows observing RNAP occupancy and thus active transcription on the genome. We did not observe a strong correlation between the RNA-seq and ChIP-seq data over coding sequence of down-regulated flagellar genes in the *lptD4213* mutant (Figure 4B), possible because the antibodies to *E. coli* RNAP core subunit are not suitable to pull-down core RNAP from *PA* that is in the elongation phase of transcription. However, we observed reduced RNAP occupancy using antibodies to the RpoD component that captures RNAP at the promoters (i.e. during transcription initiation phase) in region that are predicted to contain the promoters of this operons (red brackets on Figure 4B), which is compatible with reduced transcription. Nevertheless, we think that there was a general

problem with these experiments as less than 0.5% of genes showed fold change values bigger than 1.5 or smaller than -1.5 after VNC exposure. The fact that we observed peak on potential promoter regions suggests that the antibody to *E. coli* RpoD was able to recognize the PA14 RpoD. Interestingly, we have previously observed in a Tn-seq experiment that disruption of flagellar genes could change PA14 fitness in presence of VNC, either positively or negatively depending on the disrupted genes. Others have shown that loss of flagellar genes or motility confers a fitness advantage to PA14 in presence of subinhibitory concentration of VNC in liquid culture[9]. Thus, we tested whether deletion of flagellar genes could protect PA14 against VNC. We deleted flagellar genes coding for the major protein of the M ring (*fliF*), for one of the 4 proteins forming the rod of the flagellar basal body (*flgC*), and for the only flagellin of PA14 (*fliC*). None of the three deletions affected VNC sensitivity of *WT* PA14 cells on solid media. However, the *flgC* deletion slightly decreased the VNC sensitivity of the *lptD4213* mutant while the *fliC* deletion had the opposite effect.

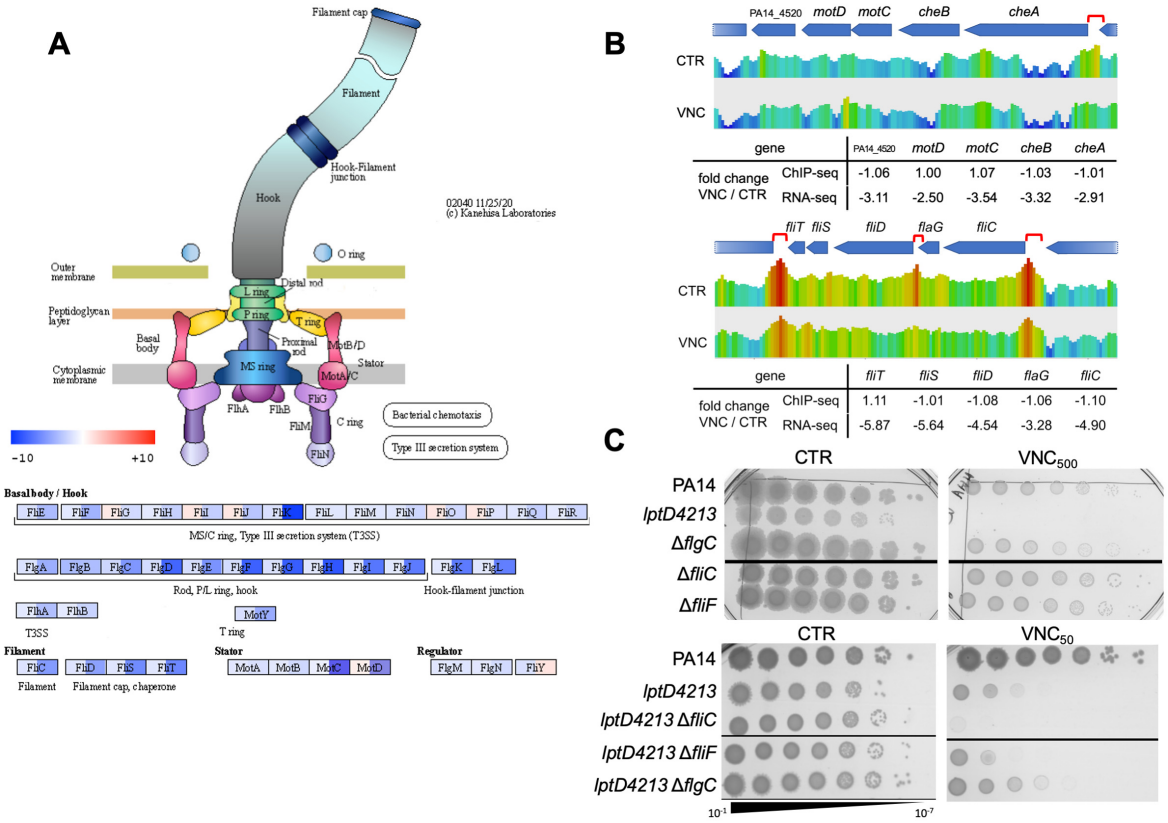


Figure 4 VNC exposure reduces transcription of most genes encoding flagellar subunits in *lptD4213* leaky mutant, and to a lesser extent in *WT* PA14, but deletion of flagellar genes does not protect *P. aeruginosa* against vancomycin. A) Graph showing the color-coded transcriptional response of genes implicated in flagellar assembly following (KEGG category pau02040, transcriptional regulators are not shown) according to RNA-seq data in wild-type PA14 (left part of each box) and l*ptD4213* mutant (right part of each box). B) Profiles of ChIP-seq performed with antibodies to core RNAP and antibodies to RpoD in PA14 *lptD4213* mutant following vancomycin exposure (VNC 100 µg/ml, 30 min) or in control conditions (CTR). Two genomic region containing genes implicated in flagellar assembly are shown. Each bar corresponds to a consecutive 50 bp window with the amount of read coded by height and color. Potential promoter regions (according to ChIP-seq data and operon annotation in pseudomonas.com) are shown with red brackets. Tables below the profiles

show the fold change of ChIP-seq read numbers for each gene coding sequence (from 50 bp after the start to 50 bp before the end) following vancomycin exposure (VNC / CTR). Fold-change data from the RNA-seq experiment is shown for comparison (VNC 200 μg/ml, 90 min). C) Upper panel, efficiency of plating assay of wild-type PA14 WT, *lptD4213,* Δ*flgC.* Δ*fliC,* and Δ*fliF* mutants in absence (CTR) or presence of 500 μg/ml VNC (VNC_{500}). Lower panel, efficiency of plating assay of wild-type PA14 WT, *lptD4213, lptD4213* Δ*fliC, lptD4213* Δ*fliF,* and *lptD4213* Δ*flgC* in absence (CTR) or presence of 50 μg/ml VNC (VNC_{50}).

The data presented here clearly support an essential role of the OM in protecting *P. aeruginosa* against VNC. Indeed, mutants with a leaky OM are highly sensitive to VNC and we previously demonstrated that efflux pumps do not contribute to the resistance of PA14 to VNC. However, the RNA-seq results showed that *WT* cells can also sense exposure to VNC and respond to it by changing their gene expression pattern, including the increased expression of the genes required for the L-Ara4N modification of the LPS which is known to increase resistance Gram-negative bacteria to COL, probably by reducing the LPS negative charge thus decreasing interaction with the positively charged COL[10]. Yet VNC is not charged and it is not clear if and how L-Ara4N modification could protect against VNC. Altogether, our results raise the following questions: 1) Could minute amount of VNC cross the OM of *WT* PA14 through OMPs or TBDRs, thus inducing a cell envelope stress response through partial inactivation of PG synthesis in the periplasm? If VNC cannot cross the OM, does it interact with and modified its property thus activating an OM stress sensor? 2) Is the transcriptional response of WT PA14 to VNC adaptative? In other words, does PA14 need changes in its cell-envelope metabolism for long-term survival and growth in presence of VNC? These questions will guide us in future work.

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