Supplemental Materials for:

Effect of vancomycin on cytoplasmic peptidoglycan intermediates and van operon mRNA levels in VanA-type vancomycin resistant *Enterococcus faecium*

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Supplemental Results

LC-MS/MS method development. Table S1 shows the optimized parameters for LC-MS/MS detection of alternative pathway intermediates in VREfm.

Table S1 Summary of optimized parameters and sensitivities for negative mode IP-LC-MS/MS detection of UDP-linked intermediates in VRE[a,b].

	t_r (min)	Q1	Q3	DP (V)	EP (V)	CE (V)	AU/pmol	LOD (fmol)[c]
UDP-Penta	12.1	1148.3	403.0	-140	-10	-60	3970	80
UDP-Penta-D-iAsp[d]	17.5	1263.3	403.0	-140	-10	-60	3970	80
UDP-Pentadepsi	15.5	1149.3	403.0	-120	-10	-62	4120	70
UDP-Pentadepsi-D-iAsp	20.5	1264.3	403.0	-100	-8	-45	2450	180
UDP-Tetra	11.0	1077.3	403.0	-125	-12	-56	3550	89
UDP-Tetra-D-iAsp[e]	13.10	1192.3	403.0	-125	-12	-56	3550	89

[a] t_r, chromatographic retention time; Q1, quadrupole 1 m/z for analyte precursor ion; Q3, quadrupole 3 m/z for analyte fragment ion; AU, area units; CE, collision energy; DP, declustering potential; EP, entrance potential; LOD, lower limit of detection; t_r, retention time.

[b] For all ions: Collision cell entrance potential = -8 V; Collisionally activated dissociation gas level, arbitrary units = medium; Source temperature = 600 °C; Curtain gas setting = 30 psi; GS1 = 70 psi; GS2 = 20 psi.

[c] Lower limits of quantification (LLOQs) were 3.3x the LOD for a particular analyte.

[d] UDP-Penta-D-iAsp MS/MS detection values were from UDP-Penta.

[e] UDP-Tetra-D-iAsp MS/MS detection values were from UDP-Tetra.

Figure S1 shows the corresponding LC-MS/MS chromatograms for these alternative pathway intermediates, with UDP-Penta included for reference.

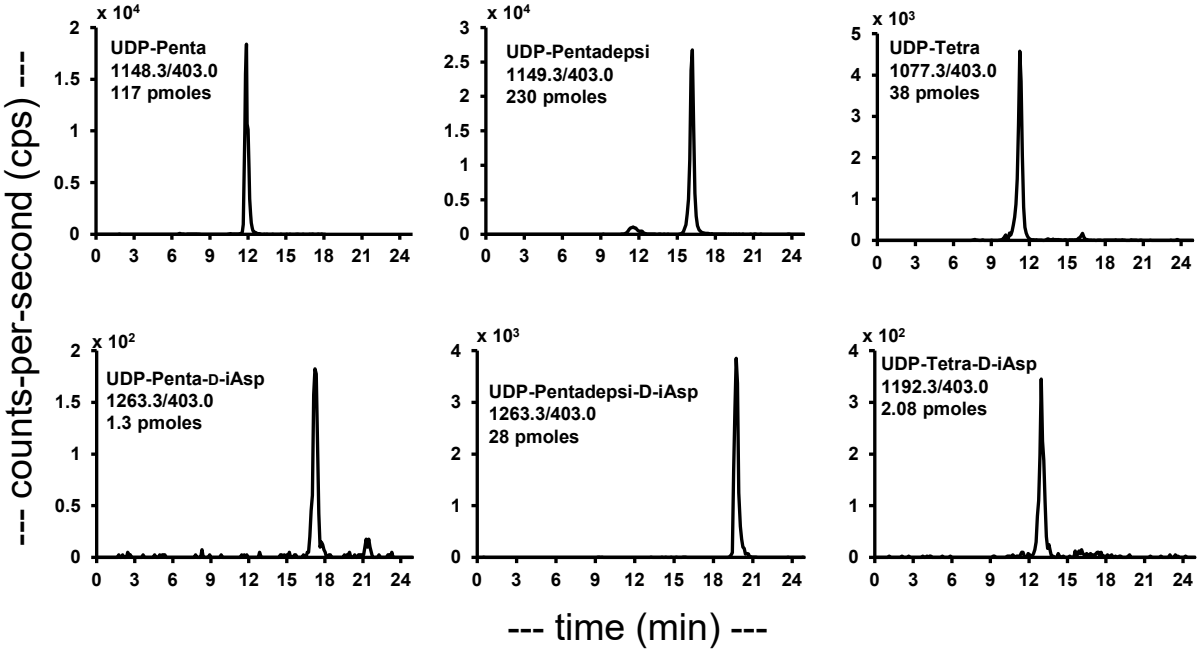

Figure S1. LC-MS/MS chromatograms of VanA type VRE specific cytoplasmic UDP cell wall intermediates. UDP-Penta included for reference.

Detailed summary of survey of vancomycin effects on CWB intermediates in *E. faecium* results (Table 1).

VSEfm-Vm v VREfm-Vm: The UDP-linked PG pathway intermediate profiles of VSEfm-Vm and VREfm-Vm have similar (+/-3-fold) levels of normal (i.e. not VanA resistance related) UDP-linked pathway intermediates. UDP-Sum (the sum of all UDP-linked intermediate concentrations) is also similar in these control samples. Most PG related amines included in this study also show similar levels between VSEfm-Vm and VREfm-Vm, with the exception of D-Ala-D-Ala, which is notably lower in VREfm-Vm than in VSEfm-Vm. MRSA-Vm shows higher amino acid levels than VSEfm-Vm and VREfm-Vm.

MRSA+1.5 v VSEfm+1.5: After vancomycin treatment for 90 minutes (1.5 h, +1.5), substantial changes in metabolite levels are apparent. As reported previously (28), MRSA+1.5 shows a dramatic increase in total UDP-linked metabolites (UDP-Sum) – particularly of the terminal cytoplasmic intermediate UDP-Penta – due to continued synthesis of UDP-linked intermediates and their accumulation. VSEfm+1.5 in contrast shows a decrease in early UDP-linked intermediates, and only a relatively modest increase in UDP-Penta and UDP-Sum, indicating that entry into the pathway is restricted in VSE in the presence of vancomycin, and also possibly that turnover of pathway intermediates continues at a reduced rate. Amines levels are also decreased in VSEfm+1.5.

VREfm-Vm v VREfm+1.5: In VREfm+1.5, most normal intermediate levels are close to their VREfm-Vm values, except UDP-Penta, which is modestly higher. Substantially increased levels of VanA-type resistance UDP intermediates are apparent in VREfm+1.5 – particularly UDP-Pentadepsi, UDP-Tetra, and D-Ala-D-Lac, which are the key intermediates for vancomycin resistance in VanA-type VREfm (Fig. 1). UDP-Pentadepsi, synthesized from D-Ala-D-Lac, is the replacement for UDP-Penta in VanA-type resistance, and UDP-Tetra is the degradation product of UDP-Penta in VanA-type resistance. D-Ala-D-Ala is decreased modestly.

VREfm+18 v VREfm+1.5 & VREfm-Vm: After extended (18 hr) vancomycin exposure (VREfm+18), the normal UDP-linked metabolite levels have partially returned towards VREfm-Vm levels compared to VREfm+1.5 with the exception of UDP-Penta, which is lower in VREfm+18 than in either the VREfm-Vm or VREfm+1.5. D-Ala-D-Ala levels are also low in VREfm+18. The unique VanA-type resistance UDP-linked intermediate levels have also dropped from their 90 min exposure levels. UDP-Sum is nearly the same in VFRfm+18 as in VREfm-vanc. These data indicate that, upon vancomycin exposure, VREfm shows an initial accumulation of both normal and alternative UDP-linked pathway intermediates, followed by a partial return of normal intermediates to their -Vm levels and a drop in the alternative intermediate levels from the higher values observed in VREfm+1.5. The level of the D-isoAsp containing intermediates is small and only detectible in vancomycin treated VREfm samples and appear unlikely to play a significant role in PG biosynthesis in VREfm.

Time course results from both VREfm and VSEfm. Time course results from both VREfm and VSEfm were plotted on a “semi-square root” x-axis to expand early time course changes.

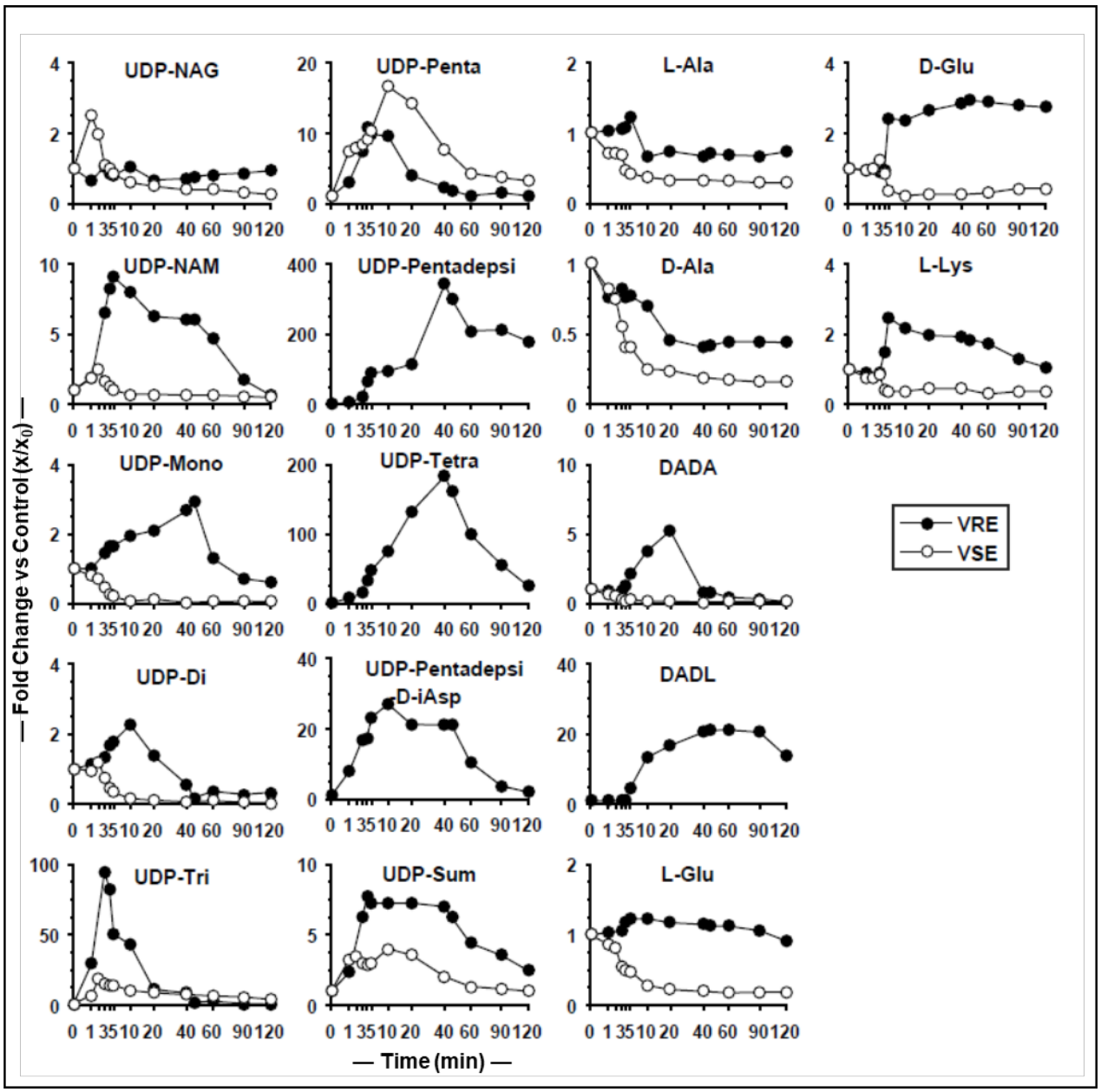

Figure S2. Time courses of VREfm PG intermediates (same data as in main text Fig. 2), and VSEfm PG intermediates plotted as fold changes vs their t_0 values (t_0 values in Table 1). The x-axis is plotted in a semi-square root form to expand early time points for easier visuallization.

Supplemental Materials and Methods

Primers used in this study for RT-qPCR (Table S2).

Table S2 Primers used for RT-qPCR of VanA mRNA transcripts.

Gene	Primer	Sequence (5′ – 3′)
VanH	F	CGATAATTAACGCCAACGTG
	R	ATTTCACACCGGCTCTCTTC
VanA	F	TAATTGAGCAGGCTGTTTCG
	R	TACTGCAGCCTGATTTGGTC
VanX	F	GATTGCTTCTATGGGACGGT
	R	CAGTTCGGTCAATATTGGGA
VanY	F	ATGCTTGGAAATACGGGTTC
	R	CCATATATTCCTCGAGAACG
VanZ	F	TTGGAGCGACAGACATAACA
	R	TGATTCATATGCTTATTGCT
16S	F	GTCAGCTCGTGTCGTGAGAT
	R	GAGTGCCCAACTGAATGATG

F – Forward primer, R – Reverse primer.