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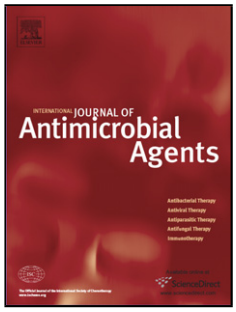

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Identification of a new gene, *vanV*, in *vanB* operons of *Enterococcus faecalis*

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ABSTRACT

Subinhibitory concentrations of vancomycin are known to induce a cell-wall stimulon in some Gram-positive pathogens, but this has never been studied in the genus *Enterococcus*. In this study, *Enterococcus faecalis* V583 strain was submitted to a subinhibitory concentration of vancomycin. DNA microarray technology was used to analyse the transcriptomic changes induced by this antibiotic. EF2292, annotated as a hypothetical protein in the *E. faecalis* V583 genome, was highly induced in response to vancomycin exposure, to similar levels as the *vanB* operon genes. We investigated further and provide evidence for co-transcription of *ef2292* with $vanY_BWH_BBX_B$ genes. It was also demonstrated that expression of *ef2292* is under the control of $vanR_BS_B$ and it is proposed to name it *vanV*. This gene was found not to be required for vancomycin resistance under the conditions tested, thus coding for another accessory protein in the *vanB* operon. *vanV* was detected in some, but not all, *E. faecalis* carrying the *vanB* operon, suggesting that this operon can have different composition amongst *E. faecalis* isolates.

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1. Introduction

Vancomycin has been used for the treatment of severe infections caused by Gram-positive bacteria for over 30 years before resistance was described. The first vancomycin-resistant enterococcal strain was isolated in 1986 [1] and since then vancomycin resistance has spread among the enterococci. In 1990, the *vanA* gene was characterised and was found to encode an inducible resistance protein, VanA, with D-Ala-D-Lac ligase activity [2]. VanH, a dehydrogenase, was subsequently found to be induced by vancomycin and to be required for vancomycin resistance. In 1992, VanR was described and it was found to be part of a two-component regulatory system, VanR–VanS, that activates a promoter inducing transcription of *vanH*, *vanA* and *vanX* (encoding a D,D-dipeptidase). The VanB phenotype was first described in 1989 [3]. The *vanB* cluster is organised and functions in a manner similar to *vanA*, differing mainly in that, unlike the *vanA* operon, it is induced by vancomycin but not teicoplanin. The *vanB* proteins, $VanH_B$, VanB and $VanX_B$, exhibit a high level of sequence identity (67–76%) with corresponding proteins of the *vanA* operon. The $VanR_BS_B$ proteins encoding the two-component signal transduction system are more distantly related to VanRS (34% and 24% identity, respectively). In both VanA- and VanB-type resistances, vancomycin resistance is due to synthesis of peptidoglycan precursors ending in the depsipeptide D-Ala-D-Lac instead of D-Ala-D-Ala [4]. Both clusters possess accessory proteins that do not contribute detectably to vancomycin resistance, including VanY and VanZ in the *vanA* cluster, and $VanY_B$ and VanW in the *vanB* cluster.

In other bacteria susceptible to vancomycin, subinhibitory concentrations of this antibiotic are able to induce several genes included in the so-called cell-wall stimulon. In the present work, in an attempt to understand the extent of the effect of vancomycin at the transcription level in *Enterococcus faecalis*, the response of V583, the prototype *vanB* strain, to a

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subinhibitory concentration of vancomycin was investigated. It was observed that upon exposure to the antibiotic, open reading frame (orf) EF2292, located immediately 3' of the *vanX*$_B$ gene, exhibited the same level of induction as the vancomycin resistance genes. This led us to ask whether EF2292 is part of the *vanB* operon and whether it contributes to vancomycin resistance. In the present study, possible co-transcription of *ef2292* with the *vanB* operon genes in *E. faecalis* V583 was evaluated by reverse transcriptase polymerase chain reaction (RT-PCR) and Northern blot hybridisation. A deletion mutant of *ef2292* was also constructed to assess its contribution to vancomycin resistance.

2. Materials and methods

2.1. Strains and growth conditions

Enterococcus faecalis strain V583 has previously been characterised in detail [3] and the sequence of its genome has been determined [5]. For DNA microarray experiments, V583Ery^S, a derivative strain of V583 susceptible to erythromycin by deletion of the EFA0007 (*ermB*) gene (kindly provided by A. Benachour, Caen, France), and V583 *vanR* insertional mutant [6] were used. Cells were grown overnight in brain–heart infusion broth at 37 °C, were diluted 20-fold and then allowed to re-grow to an optical density at 600 nm of 0.4–0.45. Vancomycin (Sigma, Steinheim, Germany) was then added to a final concentration of 0.3 mg/L. For RT-PCR and Northern blot analysis, V583 cells were grown similarly. For these two assays, 10 mg/L vancomycin were used to increase the signal and to allow better visualisation of bands in the gels. Cultures were incubated further and 5 mL samples were collected 30 min following vancomycin addition. Samples were immediately suspended in RNAprotect solution (QIAGEN, Hilden, Germany) and were centrifuged for 10 min at 4 °C.

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2.2. RNA extraction

Extraction of total RNA was performed using RNeasy mini columns (QIAGEN). DNA digestion was accomplished by adding 1 U of RNase-free DNase I (Roche, Mannheim, Germany) and incubating reactions at 37 °C for 1 h. When necessitated by residual DNA contamination, DNA digestion was repeated. Remaining RNA was further purified using the RNA Cleanup Kit (QIAGEN). Prior to PCR analysis, DNA contamination of the RNA samples was assessed in a standard (30 cycle) PCR reaction using primers targeting *ef2218*, a chromosomal gene. The primers used are listed in Table 1.

2.3. DNA microarray

The Affymetrix microarrays used were previously designed and described [8]. A full list of probe sets, including genes represented and excluded from the microarray, is available in the ArrayExpress public repository (http://www.ebi.ac.uk/) under accession no. E-MEXP-1090. Details of the algorithm used in construction of custom Affymetrix GeneChips are available at the manufacturer's website (http://www.affymetrix.com/index.affx). Three batches of RNA were obtained in three separate growth experiments to provide true biological replicates. Each set of independent RNA samples was used to perform the microarray hybridisations. cDNA synthesis, fluorescent labelling, oligonucleotide array hybridisation and preliminary data analysis were performed by the company Genome Explorations (Memphis, TN). Average relative fold changes were calculated from the average of the signal log ratio (SLR) from three separate experiments using the following equations: for an SLR of ≥0, average relative fold change = 2^{SLR}; for an SLR of <0, average relative fold change = $-1 \times 2^{(-1 \times SLR)}$.

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2.4. RT-PCR

Prior to RT-PCR, cDNA synthesis was accomplished using a Transcriptor High Fidelity cDNA Synthesis Kit (Roche) according to the manufacturer's instructions, using 1 μg of RNA as template. PCR reactions were performed using a reverse primer complementary to *ef2292* and a forward primer complementary to each of the other *vanB* operon genes ($vanY_B$, *vanW*, $vanH_B$, *vanB* and $vanX_B$). The nucleotide sequences of primers used are listed in Table 1. RT-PCR products were sequenced by Stab Vida (Lisbon, Portugal).

2.5. Northern blot analysis

For the Northern blot experiments, 20 μg of RNA was separated by electrophoresis through 1.2% agarose in a 3-(*N*-morpholino)propanesulfonic acid (MOPS)/formaldehyde gel [9]. RNA was transferred to a Hybond-N^+ membrane (Amersham Biosciences, Amersham, UK) by capillary action using 20× SSC (sodium chloride–sodium citrate solution) as the transfer buffer. RNA was ultraviolet cross-linked to the membrane by exposure to 1200 mJ/cm^2 for 2 min. Different oligonucleotide probes complementary to EF2292, the EF2292–*vanX* intergenic region, *vanX* and *vanB* were designed. Then, 0.5 pmol of each single-stranded DNA probe was labelled at the 5' end with [γ-^{32}P]ATP (Perkin Elmer, Waltham, MA) using 10 U of T4 polynucleotide kinase (Fermentas, St Leon-Rot, Germany). All probes were purified using MicroSpin™ G-50 columns (GE Healthcare, Uppsala, Sweden). Hybridisations were carried out at 42 °C with PerfectHyb™ Plus Hybridization Buffer (Sigma). When appropriate, stripping of the membranes was done following standard protocols, and membranes were re-hybridised with a different radiolabelled probe.

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Following a washing step, signals were visualised on a STORM 860 PhosphorImager (GE Healthcare).

2.6. Screening of vanV in enterococcal isolates

Eight *vanA* clinical enterococcal isolates (two *E. faecalis* and six *Enterococcus faecium*) (provided by Rosário Mato, ITQB-UNL, Oeiras, Portugal) [10] and 23 *vanB* isolates [5 *E. faecalis* (4 of which were clinical isolates) and 18 *E. faecium* (17 of which were clinical isolates)] (provided by Rob Willems, University Medical Center, Utrecht, The Netherlands) were screened for the presence of *vanV* by PCR using the primers described in Table 1.

2.7. Mutant construction

A markerless deletion mutant was constructed by double cross-over essentially as described previously [11]. Briefly, 5' and 3' flanking regions of the target gene were amplified from chromosomal DNA of V583 by PCR. The two PCR fragments were fused by PCR using the external primers and the resulting PCR fragment was cloned into the T/A cloning vector pGEM®-T (Promega, Madison, WI). The inserted PCR fragment was excised and was subsequently cloned into pG+host9 plasmid, which was then electroporated into *E. faecalis* V583. The *ef2292* double-crossover mutant was selected as described by Brinster et al. [11]. Successful target mutations were identified by PCR screening and were confirmed by Southern blot analysis.

2.8. Minimum inhibitory concentration (MIC) determination

To characterise the mutant phenotype, the MIC for vancomycin was determined by Etest (AB BIODISK, Solna, Sweden) according to the manufacturer's instructions, and by

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microdilution according to Clinical and Laboratory Standards Institute (CLSI) guidelines (http://www.clsi.org/source/orders/free/m31-a3.pdf). *Enterococcus faecalis* ATCC 29212 was used as a control strain.

2.9. Sequence analysis

The sequence of EF2292 was analysed with a number of freely available online software, namely BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/), T-COFFEE (http://www.ebi.ac.uk/Tools/msa/tcoffee/), InterProScan (http://www.ebi.ac.uk/Tools/pfa/iprscan/) and PrositeScan (http://expasy.org/tools/scanprosite/).

3. Results and discussion

The subinhibitory concentration of vancomycin used in this study did not induce a cell-wall stress response in *E. faecalis* V583. The only induced genes were those from the *vanB* operon and a hypothetical protein, EF2292. Fold-change values were 47 for $vanY_B$, 41 for $vanH_B$, 40 for *vanW*, 26 for *vanB*, 34 for $vanX_B$ and 26 for *ef2292*. This orf EF2292, induced at the same level as the vancomycin resistance genes, is located immediately upstream of the $vanX_B$ gene from the *vanB* operon. These findings suggested that EF2292 could be part of the *vanB* operon and eventually contribute to vancomycin resistance.

RT-PCR was used to determine whether *ef2292* was transcribed with the other genes of the *vanB* operon. As shown in Fig. 1a, an amplicon was obtained. Its sequence was confirmed indicating that *ef2292* is in fact co-transcribed. Therefore, it is likely that *ef2292* transcription

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occurs from the same promoter P_{YB}, since it was possible to amplify the full-length transcript. Probes from across the operon hybridised to the same transcripts in Northern blot hybridisation, indicating that they originate from the same promoter. The transcript with an approximate size of 4.6 kb is consistent with mRNA comprising *vanY$_B$WH$_B$BX$_B$ef2292* genes; the one with a size of 3.0 kb matched the *vanH$_B$BX$_B$ef2292* transcript size; and the one of 2.0 kb matches the size of a *vanBX$_B$ef2292* transcript (Fig. 1b). Further support for co-transcription of *ef2292* with other *van* genes stems from control microarray experiments using RNA from a *vanR* mutant [6]. In these assays, transcription levels both of *vanB* operon genes and *ef2292* were the same before and after vancomycin addition, confirming that neither *vanB* operon genes nor *ef2292* are induced by vancomycin if *vanR* is disrupted. Taking all these results together, and considering the generally accepted definition of an operon as a series of genes transcribed in a single mRNA, often identified by the presence of promoters and terminators, we propose a new *vanB* operon constitution for *E. faecalis* V583 as represented in Fig. 2 and to name *ef2292* as *vanV*.

To determine whether the *vanV* gene is present in other enterococcal strains, a BLAST search was performed against the 28 recently sequenced enterococcal genomes [12]. Only one positive match was found in the *E. faecalis* Merz96 genome and it exhibited 100% sequence identity. This is a blood isolate containing the *vanB* operon [8]. In Merz96, *vanV* is also located immediately upstream of the *vanX$_B$* gene, thus revealing the same *vanB* operon structure as *E. faecalis* V583. We also searched for *vanV* by PCR in *E. faecalis* (7 isolates) and *E. faecium* (24 isolates) isolates containing *vanA* (8 isolates) or *vanB* (23 isolates) operons. Only two strains yielded amplicons with the expected size (1651 bp), namely *E. faecalis* V582 (isolated from animal calf faeces) and *E. faecalis* DSM 12956 (isolated from peritoneal fluid), both carrying the *vanB* operon. Merz96 is the only *vanB E.*

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faecalis isolate amongst the 28 recently sequenced enterococcal genomes, which means that *vanV* is present in the two sequenced *vanB E. faecalis* strains belonging to different sequence types [11]. *vanV* was exclusively present in *E. faecalis* strains containing the *vanB* operon, with a prevalence of 16% (4/25 *vanB* strains) amongst *vanB* isolates and of 57% (4/7 *vanB E. faecalis*) amongst *vanB E. faecalis* isolates. It is not possible at the moment to predict whether *vanV* has survived extensive selection or whether it is an accidental rider in the *vanB* operon. In an attempt to understand the origin of *vanV*, a BLAST search was performed against intrinsically vancomycin-resistant species (such as *Lactobacillus*, *Pediococcus* and *Leuconostoc*), the producer strain *Amycolatopsis orientalis* and the vancomycin-resistant biopesticide *Paenibacillus* spp. No sequences similar to *vanV* were found. All these species produce peptidoglycan precursors terminating in lactate and possess ligase genes with homologies with either VanA [13] or VanB ligases [14,15]. Thus, the origin of *vanV* is obscure. V583 was isolated in 1988 and Merz96 in 2002, which rules out a linkage with time of isolation.

To determine whether *vanV* has a role in vancomycin resistance, the gene was deleted and the MIC value for vancomycin was determined. V583Δ*vanV* showed the same MIC value as wild-type V583 (>256 mg/L by Etest and 16 mg/L by microdilution). Both values are in accordance with previous studies on V583 strain [3]. These results indicate that deletion of *vanV* confers no phenotype related to vancomycin resistance. *vanV* is thus not required for vancomycin resistance, as is the case for $VanY_B$ and VanW from the same operon. Operons can include functionally unrelated genes, and even conserved operons often undergo rearrangements and acquire new genes, although the mechanisms remain unclear. Price et al. [7] suggest that appending of a new gene to the end is more likely than

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additions to the beginning of a pre-existing operon, so the majority of genes retain the original promoter and regulation. This may be the case for *vanV*.

According to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, EF2292 (VanV) is a hypothetical protein with no paralogues in the V583 genome. BLAST results showed that the N-terminal region of the hypothetical protein EF2292 (VanV) has a high similarity with AraC family regulators. Protein sequence analysis using InterProScan revealed a helix-turn-helix domain in the C-terminal of this type of regulator that is absent from the EF2292 sequence, which shows a central transmembrane region. The common region between EF2292 and the AraC regulators was further investigated, and ScanProsite indicates it to be a leucine zipper pattern present in many regulatory proteins. The leucine side chains facilitate dimerisation and are presumed to interact with effector molecules. It is possible that *vanV* is involved in transcriptional regulation of *vanB* or other genes.

In conclusion, the new *vanV* gene present in some *vanB* operons of *E. faecalis* isolates was found to be co-transcribed with $vanY_BWH_BBX_B$ genes and under the control of $vanR_BS_B$. VanV is not required for vancomycin resistance under the conditions tested, thus coding for another accessory protein in the *vanB* operon. Future work on this protein is needed to clarify its function. A thorough screen of *vanV* presence in large samples of vancomycin-resistant enterococci carrying the *vanB* operon may also shed some light on the origin of this protein.

Acknowledgments

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Competing interests

None declared.

Ethical approval

Not required.

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Fig. 1. (a) Agarose gel of the reverse transcriptase polymerase chain reaction (RT-PCR) products corresponding to the transcripts, 30 min post-vancomycin induction. Transcripts were obtained using the reverse primer for *ef2292* and the forward primer for *vanY*, *vanW*, *vanH*, *vanB*, *vanX* and *ef2292* (lanes 1–6, respectively). Lane 7 corresponds to a 1 kb ladder (Invitrogen, Carlsbad, CA). (b) Northern blot analysis. mRNA transcripts from *vanB* operon genes were detected using probes for *ef2292*, the intergenic region (IR) between *ef2292* and *vanX*, *vanB* and *vanX*. Sizes of the transcripts were estimated based on the HyperLadder I (Bioline, London, UK).

Fig. 2. New composition of the *vanB* operon of *Enterococcus faecalis* V853, including the *vanV* gene. Using software available online, no promoter regions were predicted for the region upstream of *vanV* and downstream of P_{YB}. According to the predicted terminators tool (http://www.jcvi.org), there is a terminator downstream of *ef2298* ($vanS_B$), consistent with that described in the literature, but no terminators are predicted downstream of *ef2293* ($vanX_B$) or *ef2292* (*vanV*).

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Table 1

Primers designed for reverse transcriptase polymerase chain reaction (RT-PCR) and mutant construction, and oligo probes designed for Northern blot hybridisation

Primer or probe [a]	Sequence (5'→3')
Primers (RT-PCR)	
Forward *vanY*$_B$	TGAATCATCACAAACGGCATA
Forward *vanW*	ATGAACAGAAAAAGATTGACACAGC
Forward *vanH*$_B$	ACTGGGGGATATGACTGTGG
Forward *vanB*	GCA CAT GGA GCG TAA CAA TAT CG
Forward *vanX*$_B$	GGGATGCCAAGTACGCTACA
DNA contamination	An internal fragment was amplified using a forward primer CGAAGGGGAATACGAAGAGTT and a reverse primer GGGCATGCTTGGTACGCAC
Mutant construction	
ef2292 internal fragment	An internal fragment was amplified using a forward primer TCTGCTTTATTATGCGGGCG and a reverse primer GAATCAGCAGAGCAAGAACG (expected size of 282 bp)
ef2292 deletion	Upstream fragment was amplified using TTCGGGCTGTGAGGTCGG with CCTTCAACGAAAACCCGAATCAAAGCAGATTCCATCAGCGC (expected size 1131 bp), and the downstream fragment was amplified using GATTCGGGTTTTCGTTGAAGG with TTTGAAGCAGGCGAGTTACC (expected size 1163 bp)
ef2292 deletion confirmation	The primers ACGGAAGAACTAAACGCTGC and CCGAATGTCCTGTTTCAAGC were used to screen the mutant clones and confirm the deleted clones (1651 bp [b]/1363 bp [c])
Probes	
EF2292	CGCCCGCATAATAAAGCAGATTCCATCAGCGC
IR_EF2292/vanX	CTGATGAAATACTAAGTAATTTGTAGCCAACTTATAC
vanX	GGTTTTCCCGTGAAGTTATCCCATGTAGCGTAC
vanB	CATGTTCCTCCGAGCAACCGCCGAAGATAATTGCGAC

IR, intergenic region.

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[a] All the primers and probes used were designed in this study, except the forward primer for the *vanB* gene [7].

[b] Expected size for the *vanV* wild-type sequence.

[c] Expected size for the deleted *vanV*.

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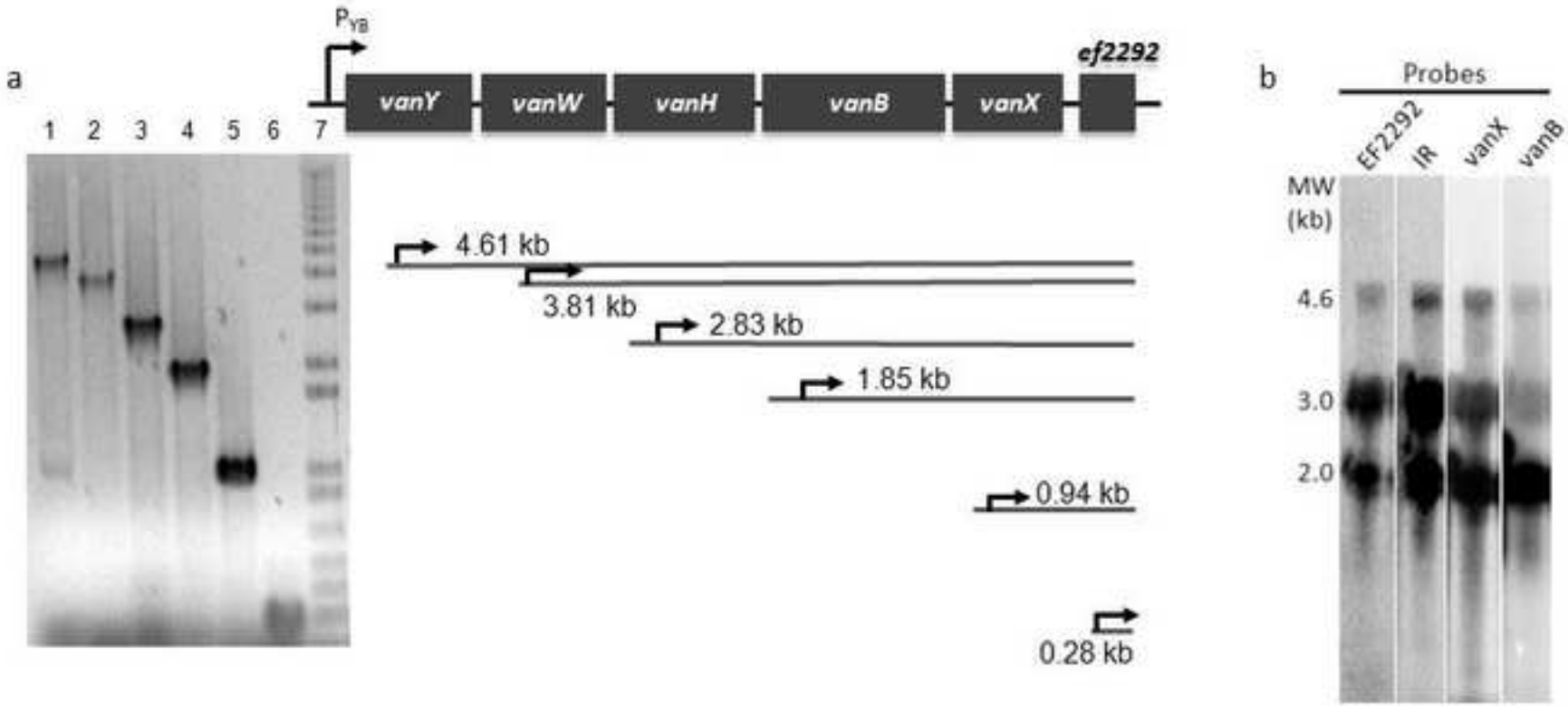
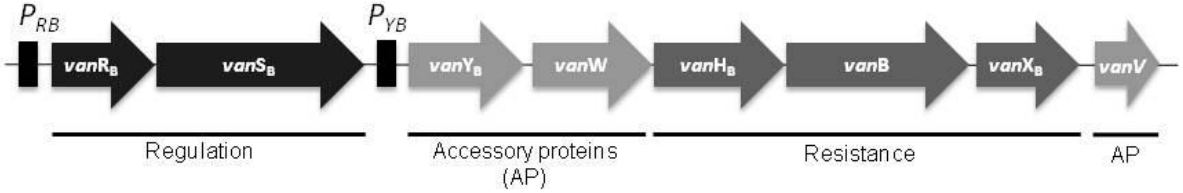
Edited Figure 1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