Competition between VanU G Repressor and VanR G Activator Leads to Rheostatic Control of vanG Vancomycin Resistance Operon Expression

Florence Depardieu, Vincent Mejean, Patrice Courvalin

To cite this version:
Florence Depardieu, Vincent Mejean, Patrice Courvalin. Competition between VanU G Repressor and VanR G Activator Leads to Rheostatic Control of vanG Vancomycin Resistance Operon Expression. PLoS Genetics, Public Library of Science, 2015, 11 (e1005170), 10.1371/journal.pgen.1005170. hal-01216338

HAL Id: hal-01216338
https://hal-amu.archives-ouvertes.fr/hal-01216338
Submitted on 16 Oct 2015

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Distributed under a Creative Commons Attribution 4.0 International License
Competition between VanUG Repressor and VanRG Activator Leads to Rheostatic Control of vanG Vancomycin Resistance Operon Expression

Florence Depardieu¹, Vincent Mejean², Patrice Courvalin¹ *

¹ Unité des Agents Antibactériens, Institut Pasteur, Paris, France, ² Laboratoire de Bioénergétique et Ingénierie des protéines, Aix Marseille Université, Marseille, France

* patrice.courvalin@pasteur.fr

Abstract

Enterococcus faecalis BM4518 is resistant to vancomycin by synthesis of peptidoglycan precursors ending in D-alanyl-D-serine. In the chromosomal vanG locus, transcription of the resistance genes from the PYG resistance promoter is inducible and, upstream from these genes, there is an unusual three-component regulatory system encoded by the vanURSG operon from the PUG regulatory promoter. In contrast to the other van operons in enterococci, the vanG operon possesses the additional vanUG gene which encodes a transcriptional regulator whose role remains unknown. We show by DNase I footprinting, RT-qPCR, and reporter proteins activities that VanUG, but not VanRG, binds to PUG and negatively autoregulates the vanURSG operon and that it also represses PYG where it overlaps with VanRG for binding. In clinical isolate BM4518, the transcription level of the resistance genes was dependent on vancomycin concentration whereas, in a ΔvanUG mutant, resistance was expressed at a maximum level even at low concentrations of the inducer. The binding competition between VanUG and VanRG on the PYG resistance promoter allowed rheostatic activation of the resistance operon depending likely on the level of VanRG phosphorylation by the VanSG sensor. In addition, there was cross-talk between VanSG and VanR’G, a VanRG homolog, encoded elsewhere in the chromosome indicating a sophisticated and subtle regulation of vancomycin resistance expression by a complex two-component system.

Author Summary

Various modes of gene regulation coexist in cells. One corresponds to the “switch on/off” mechanism in which the regulator induces the promoter to a defined level. In another mechanism, the regulator activates the promoter to various levels according to the intensity or the nature of an input signal. In this study, we show that in VanG-type vancomycin resistant Enterococcus faecalis a repressor (VanUG) allows rheostatic expression of a target...
resistance promoter by competing with a response regulator (VanR_{C}) which otherwise acts together with a sensor (VanS_{C}) by a "switch on/off" mechanism as part of a two-component regulatory system. Unusually, both regulators are encoded in the same operon.

Introduction

Vancomycin-resistant enterococci are a major cause of nosocomial infections and an important public health problem because the treatment options for the infections they cause are very limited [1]. Vancomycin, which can be the only antibiotic effective against multiresistant clinical isolates, acts by binding to the C-terminal D-alanyl-D-alanine (D-Ala-D-Ala) residues of peptidoglycan precursors blocking the extracellular steps in peptidoglycan synthesis [2]. Resistance in Enterococcus is mediated by nine types of operons that produce modified peptidoglycan precursors ending in D-Ala-D-Lac (vanA, -B, -D, and -M) or D-Ala-D-Ser (vanC, -E, -G, -L, and -N) to which vancomycin bind with a low affinity and from the elimination of the high affinity precursors ending in D-Ala-D-Ala [3–6].

Expression of the vancomycin resistance operons is regulated by VanS/VanR-type two-component signal transduction systems composed of a membrane-bound histidine kinase (VanS-type) and a cytoplasmic response regulator (VanR-type) that acts as a transcriptional activator [3]. The sensors modulate the levels of phosphorylation of the regulators. In the presence of vancomycin, VanS acts primarily as a kinase that autophosphorylates and transfers its phosphate to VanR. Phosphorylated VanR binds to the promoters upstream from the vanRS regulatory and resistance operons leading to increased transcription of the regulatory and resistance genes [7–9]. The phosphatase activity of VanS-type sensors is required for negative regulation of the resistance genes in the absence of vancomycin preventing accumulation of VanR-type regulators phosphorylated by acetylphosphate or by kinases encoded by the host chromosome [7, 10].

VanG-type Enterococcus faecalis clinical isolates from Australia and Canada are distinct from other Van-type enterococci. The chromosomal vanG cluster (Fig 1) confers resistance to vancomycin (MICs, 16 μg/ml) by inducible synthesis of precursors ending in D-Ala-D-Ser [11]. It contains the vanYG, WG, GXYG, TG resistance genes, the last three strictly required for resistance encode, respectively, a VanG ligase to synthesize D-Ala-D-Ser, a VanXYG D,D-carboxypeptidase to hydrolyse D-Ala-D-Ala, and a VanTG membrane bound serine racemase to produce D-Ser (Fig 1). As opposed to the other van gene clusters, the vanG regulatory operon contains three genes, vanUG, vanRG, and vanSG, encoding a "three component" regulatory system (Fig 1). Additional gene vanUG encodes a transcriptional regulator belonging to the Xre protein family and of unknown function. The vanURSG genes are co-transcribed, even in the

![Fig 1. Schematic representation of the vanG operon.](image)

Open arrows represent coding sequences and indicate direction of transcription. The regulatory genes are in red, the resistance genes in blue and accessory genes in green. The additional regulatory gene, vanUG, is in yellow. The vertical bar in vanYG indicates a frameshift mutation leading to a truncated protein.

doi:10.1371/journal.pgen.1005170.g001
absence of vancomycin, from the \( P_{UG} \) regulatory promoter, whereas transcription of the resistance genes is inducible and initiated from the \( P_{YG} \) resistance promoter [11].

Cryptic \( \text{vanG} \)-like operons are common in \textit{Clostridium difficile}, a major human pathogen which is a target for vancomycin, and a \( \text{vanU}_{G} \) gene encoding a protein identical to \( \text{VanU}_{G} \) was found in a clinical isolate (GenBank No AVLW01000050). A \( \text{vanU}_{G} \)-like protein (GenBank No \( \text{YP002939420}) \), 79% identical with \( \text{VanU}_{G} \), was detected in an \textit{Eubacterium} associated with a two-component system controlling an ABC-type transporter and a protein (GenBank No \( \text{YP007781704}) \) with 76% identity was reported in \textit{Ruminococcus bromii} associated with a CheY related regulator and a partial \( \text{vanG} \) operon. These regulators have not been studied.

We report the role of \( \text{VanU}_{G} \) in the transcription of the \( \text{vanG} \) operon in \textit{E. faecalis}. We show that \( \text{VanU}_{G} \) binds to the \( P_{UG} \) regulatory and \( P_{YG} \) resistance promoters and negatively regulates the \( \text{vanUR}_{SG} \) regulatory and resistance operons. In contrast, \( \text{VanR}_{G} \) binds only to \( P_{YG} \). It thus appears that, upon induction by vancomycin, the \( \text{VanS}_{G} \) sensor phosphorylates \( \text{VanR}_{G} \) which competes and displaces \( \text{VanU}_{G} \) from \( P_{YG} \) leading to transcription of the resistance operon in a dose dependent manner. Thus, rheostatic regulation of resistance gene expression results from binding of a repressor and an activator encoded in a single operon to the same promoter.

### Results

\textbf{VanU}_{G} \text{ but not VanR}_{G} \text{ binds to the } \text{P}_{UG} \text{ regulatory promoter}

Primer extension of the region upstream from \textit{vanU}_{G} indicated that, irrespective of induction, the transcriptional start site for \textit{vanUR}_{SG} was located 22 bp upstream from the translation initiation codon of \textit{vanU}_{G} [11]. The \( P_{UG} \) promoter consists of -35 and -10 regions corresponding to \textit{\delta}70 recognition sequences separated by 17 bp (Fig 2A). To determine if \( \text{VanU}_{G} \) and \( \text{VanR}_{G} \) bind to the \( P_{UG} \) regulatory promoter region and to identify putative specific binding sites, DNaseI footprinting experiments were carried out. A radiolabeled PCR probe corresponding to positions -247 to +110 relative to the transcription initiation site of \( P_{UG} \) was incubated with increasing amounts of purified \( \text{VanU}_{G} \), \( \text{VanR}_{G} \), and \( \text{VanR}_{G} \) phosphorylated (\( \text{VanR}_{G-P} \)) by acetyl phosphate. The \( P_{UG} \) region protected by \( \text{VanU}_{G} \) depended on the protein concentration, extending from -70 to -20 (positions relative to the transcription initiation site) overlapping the -35 sequence at a low concentration (Fig 2B, lane 6) and from -70 to +10 at higher concentrations (Fig 2B, lanes 7 and 8). The region (-70 to -20) contained two adjacent imperfect palindromic sequences likely corresponding to the binding motifs of \( \text{VanU}_{G} \) (Fig 2A). As opposed to the wild-type fragment, two DNA fragments containing double mutations in the imperfect dyad symmetry operator of \( P_{UG} \) were not retarded by \( \text{VanU}_{G} \), indicating a key role in \( \text{VanU}_{G} \) binding (S1 Fig). The appearance of several DNase I hypersensitive sites (Fig 2B) corresponding to bending of the DNA duplex suggested binding of two \( \text{VanU}_{G} \) monomers or dimers. This is consistent with the presence of two inverted repeats in the \( P_{UG} \) region (Fig 2A) and with the two-step gel retardation (S1 Fig). In contrast to \( \text{VanU}_{G} \), \( \text{VanR}_{G} \), and \( \text{VanR}_{G-P} \) did not bind to the \( P_{UG} \) promoter.

\textbf{VanU}_{G} \text{ acts as a repressor of the } \text{P}_{UG} \text{ regulatory promoter}

The \( \text{vanG} \) operon is part of a large genetic element and is transferable from \textit{E. faecalis} BM4518 to \textit{E. faecalis} JH2-2 from chromosome to chromosome [11]. Since clinical isolate BM4518 is not transformable, we studied the \( \text{vanUR}_{SG} \) system in transconjugant BM4522 (JH2-2::\( \text{vanG} \)) (S1 Table). To determine the role of \( \text{VanU}_{G} \) on \( P_{UG} \), the \( \text{vanU}_{G}, \text{vanR}_{G} \) and \( \text{vanS}_{G} \) genes of BM4522 were inactivated individually by in-frame deletions leading to BM4720(\( \Delta \text{vanU}_{G} \)), BM4721(\( \Delta \text{vanR}_{G} \)), and BM4722(\( \Delta \text{vanS}_{G} \)). Transcription of the regulatory genes was quantified by RT-qPCR. In BM4522, low level transcription occured at similar levels without and with
various concentrations of vancomycin indicating that the $P_{UG}$ promoter was not inducible by vancomycin (Fig 2C). In the absence of $vanU_G$, $vanR_G$ and $vanS_G$ were transcribed in the absence or presence of vancomycin at higher level ($\approx 5$-fold) from $P_{UG}$ indicating that VanU_G acted as a repressor on this promoter region (Fig 2D). In the absence of $vanR_G$ or $vanS_G$, transcription of the regulatory genes remained unchanged even in the presence of vancomycin.

To confirm regulation of $P_{UG}$ by VanU_G, the $vanURSG$ genes were cloned into vancomycin susceptible Escherichia coli NR698 [12] under the control of promoter $P_{spank}$ upstream from
$P_{UG}$ fused to a chloramphenicol acetyltransferase (CAT) reporter gene, the two promoters being separated by a transcription terminator (term) (Table 1). Subsequently, each of the three genes was inactivated. E. coli RNA polymerase bound to the $P_{UG}$ promoter (S2A Fig) which was active in the new host, in the presence or in the absence of vancomycin (Table 1). CAT was produced at a maximum level in the absence of vanUG by plasmids pAT952($P_{spank}termP_{UG}cat$), pAT966($P_{spank}vanRGtermP_{UG}cat$), and pAT969($P_{spank}vanRSGtermP_{UG}cat$) (Table 1). In contrast, in the presence of VanUG, CAT production was decreased to similar basal levels by plasmids pAT965($P_{spank}vanUGtermP_{UG}cat$), pAT967($P_{spank}vanURGtermP_{UG}cat$), and pAT968 ($P_{spank}vanURSGtermP_{UG}cat$) (Table 1). These results confirmed that VanUG acts as a strong repressor on the $P_{UG}$ promoter.

The VanRGSG two-component system is functional

Transcription of the resistance genes is under the control of VanURSG and, as discussed above, VanUG negatively autoregulates vanURSG transcription from the $P_{UG}$ regulatory promoter. To determine if VanRG and VanSG acted as a two-component system and to study the putative interaction of VanUG with these proteins, VanUG, VanRG, and the cytoplasmic histidine kinase domain of VanSG were purified as C-terminal His-tag proteins (S1 Table). VanSG autophosphorylated in the presence of $[\gamma^{32P}]$-ATP (Fig 3A). When incubated with purified VanUG or VanRG, phosphorylated VanSG transferred its phosphate group to VanRG (Fig 3B) but not to VanUG (Fig 3E). Phosphorylation of VanRG was fast and efficient, occurring in less than a minute. To test the phosphatase activity of VanSG, hydrolysis of VanRG-P over time was analysed in the absence or in the presence of VanSG. Purified $[32P]$-VanSG was stable in vitro for at least 30 min and then dephosphorylated slowly (Fig 3C); addition of purified VanSG increased dephosphorylation only slightly (Fig 3D–3G). These results indicate that VanRSG was functional and had characteristics similar to those of other VanRS-type two-component systems [7, 9] and that VanUG did not affect phosphorylation nor dephosphorylation of VanRG and VanSG (Fig 3E and 3F).

### Table 1. CAT specific activities of $P_{UG}$ promoter in E. coli NR698.

| Plasmid                          | Uninduced | Vancomycin |
|----------------------------------|-----------|------------|
| pDR111 ($P_{spank}$)             | 8 ± 4     | 11 ± 5     |
| pAT949 ($P_{spank}cat$)          | 360 ± 13  | 406 ± 22   |
| pAT950 ($P_{spank}termcat$)      | 80 ± 1    | 91 ± 5     |
| pAT964 ($P_{spank}vanUtermcat$)  | 65 ± 5    | 64 ± 6     |
| pAT952 ($P_{spank}termP_{UG}cat$)| 2023 ± 196| 2156 ± 105|
| pAT965 ($P_{spank}vanUGtermP_{UG}cat$) | 134 ± 15 | 172 ± 12   |
| pAT966 ($P_{spank}vanRGtermP_{UG}cat$) | 1856 ± 125 | 2064 ± 269 |
| pAT967 ($P_{spank}vanURGtermP_{UG}cat$) | 159 ± 13 | 146 ± 14   |
| pAT968 ($P_{spank}vanURSGtermP_{UG}cat$) | 115 ± 13 | 109 ± 13   |
| pAT969 ($P_{spank}vanRSGtermP_{UG}cat$) | 1557 ± 64 | 1478 ± 100 |

- a Results are expressed in nanomoles of product formed per minute and per milligram of protein in S100 extracts. Induction was performed with vancomycin (0.25 μg/ml). Data are means ± standard deviation obtained from a minimum of three independent extracts.
- b The $P_{spank}$ promoter is constitutive due to low expression in the absence of induction by IPTG.
- c term corresponds to the T4 transcription terminator.

doi:10.1371/journal.pgen.1005170.t001
VanUG and VanRG bind to overlapping sites of the \( P_{YG} \) resistance promoter

To study the putative binding of VanUG and VanRG to the \( P_{YG} \) region and to identify specific binding sites, DNaseI footprinting experiments were carried out. The inducible \( P_{YG} \) promoter is composed of -35 (AAAACA) and -10 (TACAAT) regions separated by 16 bp which have similarity with \( \delta_{70} \) recognition sequences, although the -35 sequence is not conserved consistent with the fact that the promoter is positively regulated (Fig 4B). Analysis of the \( P_{YG} \) region revealed three 12-bp directly repeated VanRG binding motifs and a deduced consensus sequence (T/C)CGTANGAAA(T/A)T was analogous to that in the \( PR \) and \( PH \) vanA operon.
promoters [13]. In the \( P_{UG} \) region, similar sequences were not found (Fig 2A) which could explain lack of VanRG binding. The radiolabeled probe corresponding to positions -163 to +69 relative to the transcription initiation point of the \( P_{YG} \) promoter and containing the three conserved sequences was incubated with increasing amounts of purified VanUG, VanRG, and VanRG-P (Fig 4). The three proteins protected in a concentration-dependent manner an overlapping DNA region that included the three direct repeats. The \( P_{YG} \) region protected by VanUG was much larger than that by VanRG and VanRG-P extending from -110 to -3 and overlapped the -35 sequence at 0.2 and 1\( \mu \)M (Fig 4A, lanes 17 and 18). The \( P_{YG} \) region protected by VanRG and VanRG-P extended from -100 to -56 at low concentration (Fig 4A, bracket I, lanes 3 and 8) and from -100 to -43 at higher concentrations (Fig 4A, bracket II, lanes 4 and 5, and 9 and 10). There were three binding motifs a, b, and c with different affinities for VanRG and VanRG-P in the \( P_{YG} \) promoter region (Fig 4). Only a slight difference in affinity in favor of VanRG-P at 0.2\( \mu \)M was noted for the "a" site (Fig 4A, lane 2) compared with VanRG which could be due to inefficient phosphorylation of VanRG by acetylphosphate. VanRG and VanRG-P bound to the a and b sites (Fig 4A, lanes 2, 3, and 8) with higher affinity than to the c site (Fig 4A, lanes 4 and 5, and 9 and 10), whereas VanUG bound to this DNA region with the same affinity (Fig 4A).

VanUG allows rheostatic expression of the resistance genes

To study the consequences of the binding of VanUG and VanRG to overlapping regions of \( P_{YG} \) on the expression of the resistance genes, the VanTG serine racemase was used as a reporter (Fig 5). In clinical isolate BM4518 and transconjugant BM4522, synthesis of the serine racemase was dependent on the concentration of vancomycin (Fig 5). In contrast, in BM4720(\( \Delta \)vanUG), the resistance operon was expressed at its maximum even at low concentrations of vancomycin. These results suggested that VanUG acts as a repressor of \( P_{YG} \) and that, in its absence, there is no fine-tuning of resistance expression from this promoter. Thus, modulation of transcription by vancomycin was due to the phosphorylation level of VanRG mediated by VanSC provided that VanUG was present. Surprisingly, as in the wild-type strain, induction was dependent on the concentration of the inducer in BM4721(\( \Delta \)vanRG) (Fig 5). This could be accounted for by the presence of a VanR homolog in the host. In fact, we found, in both \( E.\) faecalis BM4518 and transconjugant BM4522 which were entirely sequenced (GenBank N°PRJNA245745), a gene specifying a VanRG protein with 65% identity with VanRG (S3A Fig). In BM4722(\( \Delta \)vanSC) there was no synthesis of VanTG in the presence of vancomycin indicating that VanRG and VanRG-P are not phosphorylated in the absence of VanSC. Double mutant BM4723(\( \Delta \)vanRG, \( \Delta \)vanRG) derived from \( E.\) faecalis BM4721(\( \Delta \)vanRG) was susceptible to vancomycin (MIC, 1\( \mu \)g/ml) and VanTG production was no longer inducible by vancomycin, indicating cross-talk between VanSC and VanRG (Fig 5). To avoid interference by this regulator, transcription from the \( P_{YG} \) promoter was studied in \( E.\) coli NR698 since \( E.\) coli RNA polymerase was able to bind to this promoter (S2B Fig). The \( vanURSG, vanRSG, \) and \( vanUSG \) genes were cloned under the control of \( P_{spank} \) transcribed from the \( P_{YG} \) transcriptionally fused to a cat gene generating pAT970 (\( P_{spank}vanURSGtermPYGcat \)), pAT971 (\( P_{spank}vanRSGtermPYGcat \)), and pAT972 (\( P_{spank}vanUSGtermPYGcat \)). In the absence of VanUG, induction by vancomycin led to similar levels of CAT synthetase in the strain harboring pAT971 (\( P_{spank}vanRSGtermPYGcat \)) whatever the concentration of the inducer, whereas with pAT970 (\( P_{spank}vanURSGtermPYGcat \)) CAT production depended on the vancomycin concentration (Table 2). These results confirmed that, in the presence of vancomycin, VanUG is required for rheostatic gene transcription from \( P_{YG} \) and that VanRG phosphorylation is essential for expression of the resistance genes since, in the absence of this regulator in pAT972 (\( P_{spank}vanUSGtermPYGcat \)), the level of CAT activity was low, both
without (74 U ± 9) and with (104 U ± 13) vancomycin (0.30 μg/ml). In the absence of vancomycin, CAT activity was lower in *E. coli* producing vanUG encoded by pAT970 (P<sub>spankvanURSG</sub>termPYGcat) than in its counterpart harboring pAT971 (P<sub>spankvanRSG</sub>termPYGcat). This confirms that VanUG acts as a repressor on the P<sub>YG</sub> resistance promoter (Table 2).
VanUG and VanRG compete for binding to the $PYG$ resistance promoter

Since VanUG and VanRG bound at overlapping sites of $PYG$, to assess a possible effect of VanRG on the binding of VanUG, we performed DNaseI footprinting assays on the labeled $PYG$ probe with purified VanRG and VanUG (Fig 6). Low and medium concentrations (64 nM and 128 nM) of VanUG which allow binding to $PYG$ were tested with increasing concentrations of VanRG. Upon addition of VanRG, the binding profile of VanUG faded while that of VanRG appeared and increased in a dose dependent manner (Fig 6A). In the reverse experiment two appropriate concentrations of VanRG were challenged by increasing concentrations of VanUG and the binding of VanRG decreased also in the presence of VanUG (S4 Fig). In summary, VanUG alone did not allow transcription of the resistance genes (Fig 6B). It thus appears that at a low concentration of vancomycin there was competition between VanUG and VanRG, the latter being partially phosphorylated, transcription of $vanYGWGGXYGTG$ was low. In contrast, at high concentrations of vancomycin, VanRG was efficiently phosphorylated and able to displace VanUG leading to maximal transcription of the resistance genes from the $PYG$ promoter.

VanUG and VanRG compete for binding to the $PYG$ resistance promoter

Since VanUG and VanRG bound at overlapping sites of $PYG$, to assess a possible effect of VanRG on the binding of VanUG, we performed DNaseI footprinting assays on the labeled $PYG$ probe with purified VanRG and VanUG (Fig 6). Low and medium concentrations (64 nM and 128 nM) of VanUG which allow binding to $PYG$ were tested with increasing concentrations of VanRG. Upon addition of VanRG, the binding profile of VanUG faded while that of VanRG appeared and increased in a dose dependent manner (Fig 6A). In the reverse experiment two appropriate concentrations of VanRG were challenged by increasing concentrations of VanUG and the binding of VanRG decreased also in the presence of VanUG (S4 Fig). In summary, VanUG alone did not allow transcription of the resistance genes (Fig 6B). It thus appears that at a low concentration of vancomycin there was competition between VanUG and VanRG, the latter being partially phosphorylated, transcription of $vanYGWGGXYGTG$ was low. In contrast, at high concentrations of vancomycin, VanRG was efficiently phosphorylated and able to displace VanUG leading to maximal transcription of the resistance genes from the $PYG$ promoter.

Table 2. CAT specific activities of $PYG$ promoter in $E. coli$ NR698.

| Plasmid | 0    | 0.2  | 0.3  | 0.4  |
|---------|------|------|------|------|
| pAT970  ($P_{spank}\text{vanURSGtermPYGcat}$) | 264 ± 23$^a$ | 566 ± 54 | 797 ± 64 | 1283 ± 118 |
| pAT971  ($P_{spank}\text{vanRSGtermPYGcat}$) | 544 ± 48 | 1585 ± 115 | 1556 ± 162 | 1487 ± 142 |

$^a$ Results are expressed in nanomoles of product formed per minute and per milligram of protein in cytoplasmic extracts. Data are means ± standard deviation obtained from a minimum of three independent extracts.

$^b$ The $P_{spank}$ promoter is constitutive due to low expression in the absence of induction by IPTG.

$^c$ term corresponds to the T4 transcription terminator.
The presence of \textit{vanUG} reduces the fitness cost associated with expression of VanG-type resistance.

To study the role of VanUG in this sophisticated resistance mechanism, the fitness cost of BM4720(\textit{ΔvanUG}) compared with that of BM4522 in monocultures in the absence and in the presence of vancomycin (1 \(\mu\)g/ml) was analysed by determination of the growth rates (Table 3). The results showed that the growth rates of both strains were indistinguishable in the absence of vancomycin indicating that non induced VanG-type resistance is not costly for the host. In contrast, in the presence of vancomycin, the relative growth rate of BM4720(\textit{ΔvanUG}) (0.74) was

\[ \text{Relative growth rate} = \frac{\text{Growth rate of BM4720(ΔvanUG)}}{\text{Growth rate of BM4522}} \]

This indicates a significant fitness cost associated with the expression of VanG-type resistance in the presence of vancomycin.
significantly reduced when compared with that of BM4522 (0.93) indicating that increased expression of resistance was significantly more costly in the absence of \textit{vanU}G.

**Discussion**

Among the ubiquitous two-component regulators, VanR/VanS-type systems are one of the rare to control expression of genes mediating antibiotic resistance [3]. In the VanG-type strains, a membrane associated sensor kinase (VanSG) which detects a signal associated with the presence of vancomycin in the environment and a cytoplasmic response regulator (VanRG) that acts as a transcriptional activator are also present (Fig 1) and functional (Fig 3) but there is, in addition, a VanUG transcriptional regulator (Fig 1).

In the two main VanA- and VanB-type systems, the regulatory genes (\textit{vanRS}) and the resistance genes are transcribed from independent and coordinately regulated promoters, but VanR is the only known direct regulator of the resistance genes [3, 8, 13]. In VanG-type strains, co-transcription of \textit{vanURSG} is repressed from \textit{PUG} by VanUG (Fig 2 and Table 1) and expression of the resistance genes from \textit{PYG} is activated by VanRG and repressed by VanUG (Fig 5 and Table 2). Thus, VanUG regulates the resistance genes both directly, by binding to the \textit{PYG} promoter region (Fig 4), and indirectly by repressing synthesis of VanRGSG (Fig 5). Like other members of the XRE protein family (S3B Fig) [14–16], VanUG binds to short repeated sequences which span the promoters (Fig 2A and 2B). Unlike the VanR and VanRB proteins which bind to their own promoters [8, 13], VanRG does not regulate its own expression (Fig 2). No sequences similar to the VanRG consensus binding site are found in \textit{PUG} (Figs 2 and 4).

VanRG, as VanR and VanRB, belongs to the OmpR-PhoB subclass of response regulators that have the peculiarity to bind to their target promoters in the unphosphorylated or phosphorylated form [8, 13, 17, 18]. Phosphorylation of VanR and VanRG enhances the affinity of the proteins for their respective regulatory \textit{PR} or \textit{PRB} and resistance \textit{PH} or \textit{PYB} promoter regions allowing increased transcription of the regulatory and resistance genes [8, 13]. In VanA-type strains, VanR and VanR-P bind to \textit{PR} and \textit{PH} regions which contain a single or two 12-bp conserved sites, respectively [13]. Comparison of the sequences of the \textit{PUG} and \textit{PYG} regions with the 12-bp consensus sequence spanned by VanR and VanR-P revealed three binding sites in the \textit{PYG} region with a consensus sequence (Fig 4B) similar to that in VanA-type resistance [13]. As for the regulatory \textit{PR} and resistance \textit{PH} promoters, the positioning of these sites in \textit{PYG} was upstream from the -35 motif. VanUG, VanRG, and VanRG-P protected overlapping regions, the two latter binding to \textit{PYG a} and \textit{b} sites with a higher affinity than to the \textit{c} site (Fig 4). There are only two sites in the \textit{PH} promoter but VanR generated a more extensive footprint (80 bp for \textit{PH}) than VanRG (42bp for \textit{PYG}) likely due to higher cooperativity of VanR. Although not essential for binding in vitro, phosphorylation of VanRG increased its affinity for the \textit{PYG} resistance promoter (Fig 4). In the \textit{PUG} promoter region no sequences similar to the consensus

| Strain                  | Growth rate \textit{a} | Relative growth rate \textit{b} |
|------------------------|------------------------|---------------------------------|
| \textit{E. faecalis BM4522} | 0.027 ± 0.001          | 0.025 ± 0.001                   | 0.930 |
| \textit{E. faecalis BM4720 (ΔvanU}G\textit{)} | 0.027 ± 0.001          | 0.020 ± 0.002                   | 0.741 |

\textit{a} Exponential growth rate measured in the absence of antibiotic or in the presence of vancomycin (1μg/ml) (\textit{Vm}1); average of at least four independent experiments ± standard deviations.

\textit{b} Relative growth rate was calculated as the ratio of the growth rate of the strain induced by 1μg/ml of vancomycin versus the non induced strain.

doi:10.1371/journal.pgen.1005170.t003
were found (Fig 2A) which could explain the absence of binding of VanR\(_G\) and low-level transcription from the regulatory promoter.

In many instances, regulation of gene transcription in \textit{E. coli} occurs essentially through control of the phosphatase activity of the sensor [19, 20]. In VanA- and VanB-type strains, the level of phosphorylation of VanR and VanR\(_B\) is modulated by the kinase and phosphatase activities of the VanS and VanS\(_B\) sensors [7, 10, 21]. Phosphatase activity is critical for response regulators, such as VanR and VanR\(_B\), whose phosphorylated form is highly stable, to ensure that the protein is not permanently activated. In VanG-type strains, in the absence of VanU\(_G\), induction by vancomycin led to maximal VanT\(_G\) serine racemase (Fig 5) or CAT synthesis (Table 2) even at low concentrations of the inducer. Since in the absence of VanU\(_G\) there was no modulation of resistance genes transcription from the \(P_{YG}\) promoter, this suggests that a low amount of VanR\(_G\)-P is sufficient to induce the resistance operon. VanU\(_G\) did not modulate VanR\(_G\) and VanS\(_G\) phosphorylation (Fig 4F) and was not phosphorylated by VanS\(_G\) (Fig 4E). Surprisingly, at least in vitro, the phosphatase activity of VanS\(_G\) was not very efficient (Fig 4D) in comparison with those of VanS or VanS\(_B\) [7, 9]. Expression of VanG-type resistance was thus inducible by vancomycin due to the presence of VanU\(_G\) as opposed to direct modulation of VanR activity by VanS in the other \textit{van} operons. In the absence of vancomycin only VanU\(_G\) bound to the \(P_{YG}\) promoter; however when the concentration of vancomycin increased, VanR\(_G\) being more efficiently phosphorylated by VanS\(_G\), displaced progressively VanU\(_G\) allowing gradual transcription of the resistance genes (Fig 6) as it is likely the case with VanR\(_G\), the VanR\(_G\) homolog encoded elsewhere in the chromosome. In \textit{B. subtilis}, when both repressors SinR and SlrR are bound to the \textit{degU} promoter, they can be displaced by the response regulator DegU leading to activation of the \textit{degU} gene [22]. Also in \textit{B. subtilis}, CcpC activates aconitase gene \textit{citB} expression whereas CodY binds to its promoter and represses \textit{citB} transcription [23]; PutR which is an activator essential for transcription of the \textit{putBCP} operon for proline utilization is displaced by the CodY repressor [24].

VanU\(_G\) does not possess the characteristics of auxiliary regulators which can interact with histidine kinases, influencing signal perception and transduction. Nor does it interact with the response regulator to alter its phosphorylation status or its DNA binding ability, the recruitment of RNA polymerase on the promoter, or to sequester it through protein:protein interaction [25, 26]. The results presented here show that competition between the VanU\(_G\) repressor and the VanR\(_G\) activator for binding to the \(P_{YG}\) promoter may be responsible for the complex regulation of the resistance genes (Fig 6). This is an unusual example of rheostatic regulation of gene transcription due to binding competition between two regulators encoded in the same operon. It also elucidates an unsuspected strategy by which enterococcal clinical isolates regulate transcription of acquired genes for vancomycin resistance.

In previous work, we showed in VanB-type resistance that, despite the complex dual biochemical mechanism of resistance to vancomycin, its biological cost in enterococci is negligible when non induced, whereas a significant fitness reduction is observed when resistance is expressed in the presence of the inducer, the antibiotic itself [27]. Thus resistance is expressed exclusively when needed for bacterial survival. In VanG-type strains, tight regulation of resistance expression involves VanU\(_G\) which can thus be considered as a compensatory component, drastically reducing the biological cost associated with vancomycin resistance in the presence of antibiotic.

**Materials and Methods**

**Bacterial strains, plasmids, and growth conditions**

The origin and properties of the strains and plasmids are described in S1 Table. \textit{Escherichia coli} TOP10 (Invitrogen, Groningen, The Netherlands) and NR698 (susceptible to vancomycin) [12]
were used as a host for recombinant plasmids. *E. coli* BL21DE3 [28], in which the T7 RNA polymerase gene is under the control of the inducible lacUV5 promoter carries the pREP4 plasmid allowing co-expression of the GroESL chaperonin to optimize recombinant protein solubility [29]. *E. coli* TG1 RepA [30] was used as a host for constructions in the pAT944(pGhost9Ocat) vector (S1 Table). Kanamycin (50μg/mL) was used as a selective agent for cloning PCR products in the pCR-Blunt vector (Invitrogen). Ampicillin was used to select pUC1813 [31]. pDR111 (gift from David Rudner, Harvard University), which harbors the *Pspank* promoter between two fragments of the *B. subtilis* amyE gene, is a derivative of the Pspac-hy plasmid pJQ43 containing an additional lacO binding site to achieve a better repression in the absence of the IPTG inducer. *Pspank* is a lacI repressible IPTG inducible-promoter for gene overexpression. Spectinomycin (60μg/mL) and chloramphenicol (10μg/mL) were added to the medium to prevent loss of plasmids derived from pDR111(*Pspank*) and pAT944(*pGhost9Ocat*), respectively. Enterococcus faecalis JH2-2 is a derivative of strain JH2 that is resistant to fusidic acid and rifampin [32]. In all experiments, strains were grown in brain heart infusion (BHI) at 37°C with shaking at 110 rpm.

Promoter DNA labeling

Labeled *PUG* (357 bp) and *PYG* (233 bp) fragments were generated by PCR with BM4518 total DNA as a template and primer pairs VanG12-VanG126 and VanSG6-YG10 (S2 Table), respectively, using a combination of an unlabeled primer with an end-labeled primer (625nM) with T4 polynucleotide kinase (0.075 U/μl) (New England Biolabs) and [γ-32P]-ATP (3000 Ci/mmol) (Perkin Elmer). The PCR reactions were carried out in a 50-μl volume and the products purified as described [8].

Gel shift assay

Purified labeled PCR products corresponding to wild-type and mutated *PUG* promoter region fragments were recovered from a 6% polyacrylamide gel and used as a probe for the gel shift assay after addition of 100 μl of ammonium acetate (0.5 M) diluted in Tris buffer (10 mM, pH8.5) overnight at 37°C. The *PUG* and mutated *PUG* probes (10,000cpm each) were incubated with various concentrations of purified VanUG, regulator at 30°C for 20min in 20 μl of 50mM Tris-HCl (pH7.8) containing 20 mM MgCl2 and 0.1 mM dithiothreitol (DTT). After addition of the DNA dye solution (40% glycerol, 0.025% bromophenol blue and 0.025 xylene cyanol), the mixture was loaded on a 7.5% polyacrylamide gel in the absence of protein denaturants. The gels were dried and analysed by autoradiography.

DNase I footprinting

Complexes with the labeled promoter regions (5nM) were formed for 30 min at 30°C in 15 μl of buffer C (20 mM Hepes pH 8.0, 5 mM MgCl2, 50 mM potassium glutamate, 5 mM DTT, and 500μg/ml bovine serum albumin) using RNA polymerase of *E. coli* at 50 nM or VanUG, VanRC, or VanRC-P at increasing concentrations. For DNase I experiments, 1.5 μl of DNase I solution (1 μg ml⁻¹ in 10 mM Tris-HCl, 10 mM MgCl2, 10 mM CaCl₂, 125 mM KCl) were added and incubated at 30°C for 10s when the labeled promoter regions were alone, or for 20 s when RNA polymerase or VanUG, VanRC or VanRC-P were present in the mixture. The reaction was stopped and all the samples were extracted, precipitated, washed, resuspended, and loaded on a sequencing gel as described [8]. Protected bands were identified by comparing the migration with that of the same fragment treated for the A+G sequencing reaction [33]. The gels were analysed by autoradiography.
Quantitative real-time RT-qPCR

Enterococci grown in 100 ml of brain heart infusion in 250-ml bottles, with and without vancomycin, at 37°C with shaking at 110 rpm to OD_{600} = 0.8 were harvested. RNA was prepared using the Fast RNA ProBlue kit (MBP Biomedicals) according to the manufacturer’s protocol, treated with DNase (Turbo DNA-free, Invitrogen), and checked for the absence of contaminant DNA in a standard PCR, using the same primers as for the RT-PCR. RNA concentrations were determined by measuring absorbance with a NanoDrop2000 (ThermoScientific). cDNA synthesis and RT-qPCR were performed with a LightCycler RNA amplification kit SYBR greenI (Roche Diagnostic GmbH) in a total reaction volume of 19 μl with 0.5 μM gene-specific primers (VanG129-VanG102 for vanUG, VanRG2-VanRG10 for vanRG, VanSG2-VanSG10 for vanSG, and rpoB5-rpoB12 for rpoB) (S2 Table) according to the manufacturer’s instructions. Amplification and detection of specific products were performed using the LightCycler sequence detection system (Roche) with the following cycle profile: 1 cycle at 55°C for 20 min for the reverse transcription step, followed by 1 cycle at 95°C for 30 s, 45 cycles at 95°C for 5 s, 52°C for 15 s, and 72°C for 15 s. The level of every gene transcript was normalized relative to rpoB transcript levels.

Overproduction and purification of VanUG, VanRG, and VanSG

Plasmids pAT940(pET28ΩvanUG), pAT941(pET28ΩvanRG), and pAT942(pET28ΩvanSG) (S1 Table) were introduced into E. coli BL21λDE3/pREP4 [29]. The transformants were grown in 1 liter of LB medium in Fernbach flasks with shaking at 110 rpm at 28°C until OD600 = 0.8, IPTG (1 mM) was added to induce protein production, and incubation was pursued for 4 h. E. coli crude protein extracts were loaded on 1-ml His-Trap fast-flow columns (GE, Healthcare) equilibrated with buffer A (50mM NaH2PO4 pH 7.5, 300 mM NaCl, 30 mM imidazole) and the proteins were eluted with an imidazole gradient (30mM-500mM). Fractions were dialysed against buffer B (50mM NaH2PO4 pH 7.5, 300 mM NaCl, 50% glycerol). Protein concentration was determined using the Bio-Rad protein assay [34].

Autophosphorylation of VanSG

Autophosphorylation of VanSG (40 μg) was performed in a final volume of 100 μl of buffer A (final concentrations: 50 mM Tris-HCl, 50mM KCl and 1 mM MgCl2, pH7.5). The reaction was initiated by the addition of 5 μl of ATP (1mM final) containing 200 μCi of [γ-32P]ATP and incubated at room temperature for 1 h. ATP was removed using 500 μl Sephadex G-50 spin column equilibrated with buffer A. The reaction was quenched by the addition of 5 μl of β-mercaptoethanol-stop solution (Sigma), followed by electrophoresis on 12% NuPAGE Novex Bis-Tris gels (Invitrogen) in MOPS buffer (1X), and autoradiography.

Phosphorylation of VanUG and VanRG by VanSG

Phosphotransfer to purified VanUG and VanRG were carried out in buffer A. The reaction was initiated by the addition of 10 μl of the purified autophosphorylation reaction mixture of VanSG (40 μg) described above to a 15 μl reaction mixture containing VanUG or VanRG (55 μg each). After incubation for various periods of times at room temperature, the phosphotransfer reactions were quenched by the addition of stop solution (Sigma) followed by electrophoresis on 12% NuPAGE Novex Bis-Tris gels (Invitrogen) in MOPS buffer (1X) and autoradiography.

Phosphorylation of VanUG and VanRG by acetyl[32P]phosphate

VanUG (220 μg) or VanRG (225 μg) were incubated in 100 μl of buffer B (50 mM Tris-HCl, pH7.8, 20 mM MgCl2, 0.1 mM dithiothreitol) containing 178 pmol (3.3 μCi) of acetyl[32P]
phosphate (Hartmann Analytical, Germany) at room temperature for 60 min. Excess acetyl $^{[32]P}$phosphate was removed using Sephadex G-50 spin columns equilibrated with buffer B. Aliquots (10 μl) were withdrawn at designated time points, and the phosphorylation reactions were quenched with β-mercaptoethanol-stop solution followed by electrophoresis on 15% SDS-polyacrylamide gels and autoradiography.

**Hydrolysis of phospho-VanU<sub>G</sub> and phospho-VanR<sub>G</sub> by VanS<sub>G</sub>**

The VanU<sub>G</sub> (220 μg) and VanR<sub>G</sub> (225 μg) response regulators were labelled with acetyl$^{[32]P}$phosphate for 1 h at room temperature as described above, and 52 μg of VanS<sub>G</sub> histidine kinase were added, and incubation was pursued for various periods of times. Aliquots (10 μl) were withdrawn at designated time points and the reactions were stopped, followed by electrophoresis on 15% SDS-polyacrylamide gels and autoradiography.

**Plasmid construction**

The plasmids were constructed as follows.

**Construction of pAT940, pAT941 and pAT942.** pAT940(pET28ΩvanU<sub>G</sub>) and pAT941(pET28ΩvanR<sub>G</sub>). A 225-bp BsaI-XhoI fragment corresponding to the vanU<sub>G</sub> coding sequence amplified with UG1 and UG2 (S2 Table) and a 705-bp BsaI-XhoI fragment corresponding to the vanR<sub>G</sub> coding sequence amplified by using oligonucleotides RG1 and RG2 (S2 Table) and BM4518 [11] total DNA as a template, were cloned in the NcoI and XhoI sites of modified pET28 [35] to generate plasmids pAT940(pET28ΩvanU<sub>G</sub>) and pAT941(pET28ΩvanR<sub>G</sub>). Oligodeoxynucleotide UG1 contained a BsaI restriction site designed to generate a cohesive end compatible with NcoI and 16 bases complementary to codons 1–6 of vanU<sub>G</sub> of BM4518 (S2 Table). Oligodeoxynucleotide UG2 contained a XhoI site replacing the TGA stop codon and 21 bases complementary to codons 69–75 of vanU<sub>G</sub>. Oligodeoxynucleotide RG1 contained a BsaI restriction site designed to generate a cohesive end compatible with NcoI and 16 bases complementary to codons 1–6 of vanR<sub>G</sub> of BM4518. Oligodeoxynucleotide RG2 contained a XhoI site replacing the TGA stop codon and 21 bases complementary to codons 229–235 of vanR<sub>G</sub>.

**pAT942(pET28ΩvanS<sub>G</sub>).** A cytoplasmic portion of the vanS<sub>G</sub> gene of strain BM4518 was amplified using BM4518 total DNA as a template and primer pair SG1-SG3 (S2 Table). Oligodeoxynucleotide SG1 contained a BsaI restriction site designed to generate a cohesive end compatible with NcoI, and 16 bases complementary to codons 88–93 of vanS<sub>G</sub>. Oligodeoxynucleotide SG3 contained a XhoI site in place of the TAG stop codon and 21 bases complementary to codons 361–367 of vanS<sub>G</sub>. The 842-bp pCR product from vanS<sub>G</sub> was digested by BsaI and XhoI and cloned between the NcoI and XhoI restriction sites of plasmid pET28 to generate plasmid pAT942(pET28ΩvanS<sub>G</sub>).

**Construction of pAT944(pGhost9Ωcat).** The XbaI cassette containing the chloramphenicol acetyltransferase cat gene with its own promoter was amplified from DNA of plasmid pAT943(pUC1318Ωcat) with primers pG9CATNH2 and pG9CATCOOH (S2 Table) which contain a XbaI restriction site allowing the replacement of the XbaI fragment containing the erythromycin resistance gene in pGhost9 [36] to generate plasmid pAT944(pGhost9Ωcat).

**Construction of pAT945(pGhost9CmΩΔvanU<sub>G</sub>), pAT946(pGhost9CmΩΔvanR<sub>G</sub>), pAT947(pGhost9CmΩΔvanS<sub>G</sub>), and pAT973(pGhost9CmΩΔvanR<sub>G</sub>).** The vanU<sub>G</sub>, vanR<sub>G</sub>, and vanS<sub>G</sub> genes of the vanG operon and vanR<sub>G</sub> from BM4518 were inactivated by deletion using splicing-by-overlap extension PCR in two steps and cloned into the thermosensitive shuttle plasmid pAT944(pGhost9Ωcat) using XhoI and PstI restriction sites to generate plasmids pAT945(pGhost9CmΩΔvanU<sub>G</sub>), pAT946(pGhost9CmΩΔvanR<sub>G</sub>), pAT947(pGhost9CmΩΔvanS<sub>G</sub>), and pAT973(pGhost9CmΩΔvanR<sub>G</sub>). The primers used for the construction of
the deletant alleles and the extent of the deletions are reported in S2 Table. A Smal restriction site was added in the primers to screen for integration in the corresponding chromosomal gene. Briefly, the remnants of the vanUG, vanRG, vanSG and vanRG genes of BM4518 were first amplified from total DNA of BM4518 as a template using primers UG3-UG4 and UG5-UG6 for ΔvanUG, UG3-RG4 and RG5-RG7 for ΔvanRG, SG4-SG5 and SG6-SG7 for ΔvanSG, RG10-RG11 and RG12-RG13 for ΔvanRG, and, in a second step, the resulting PCR products were amplified with UG3 plus UG6, UG3 plus RG7, SG4 plus SG7, and RG10 plus RG13 respectively, to obtain ΔvanUG, ΔvanRG, ΔvanSG and ΔvanRG.

Construction of pAT949 and derivatives. Plasmid pAT949(pDR111ΩPspankcat) was constructed by cloning the HindIII-SphI fragment of pAT948(pUC1813Ωcat) carrying the cat cassette in pDR111(Pspank) digested with the same enzymes allowing a directional cloning of the cat reporter gene under the control of the inducible Pspank promoter.

pAT950 (pDR111ΩPspanktermcat). A 66-bp HindIII-SalI fragment corresponding to the transcription terminator of gene 32 from bacteriophage T4 [37] was amplified by PCR with oligodeoxynucleotides T4F-HindIII and T4R-SalI/NheI (S2 Table). Primer T4F-HindIII contained SalI and NheI restriction sites, the stop codon, and 14 bases complementary to the 3’ end sequence of vanUG from BM4518. The BsaI and SalI restriction sites allowed directional cloning of a 249-bp fragment of vanUG downstream from the inducible Pspank promoter and upstream from the cat reporter gene of the pAT949(pDR111ΩPspankcat) shuttle vector.

pAT951(pDR111ΩPspankvanUGcat). The vanUG gene of BM4518 was amplified using primer pair UGNH2 and UGCOOH (S2 Table) and total DNA of the corresponding strain as a template. Oligodeoxynucleotide UGNH2 contained BsaI and HindIII restriction sites, a RBS, and 6 bases complementary to vanUG including the ATG (translation initiation) codon. Oligodeoxynucleotide UGCOOH harbored Sall and Nhel restriction sites, the stop codon, and 15 bases complementary to the 3’ end of vanUG. The resulting PCR products were amplified with UG3 plus UG6, UG3 plus RG7, SG4 plus SG7, and RG10 plus RG13 respectively, to obtain ΔvanUG, ΔvanRG, ΔvanSG and ΔvanRG.

pAT952(pDR111ΩPspanktermPUGcat) and pAT953(pDR111ΩPspankvanUGPUGcat). The regulatory PUG (183 bp) promoter was amplified by PCR from BM4518 total DNA with oligodeoxynucleotides PUG1 and PUG2 (S2 Table). Primers PUG1 and PUG2 contained a NheI and a Sall restriction site, respectively, which allowed directional cloning of PUG upstream from the cat gene of pAT950(pDR111ΩPspanktermcat) to generate pAT952(pDR111ΩPspanktermPUGcat) or allowed directional cloning of PUG downstream from vanUG and upstream from the cat reporter gene of pAT951(pDR111ΩPspankvanUGcat) to generate pAT953 (pDR111ΩPspankvanUGPUGcat).

pAT954(pDR111ΩPspankvanRGcat). A 754-bp HindIII-NheI fragment corresponding to the vanRG coding sequence with its RBS, initiation and stop codons was amplified by PCR from BM4518 with oligodeoxynucleotides RGNH2 and RGCOOH (S2 Table). Primer RGNH2 contained a HindIII restriction site. Primer RGCOOH comprised Sall and Nhel restriction sites, the stop codon, and 14 bases complementary to the 3’ end of vanRG from BM4518. The HindIII and Nhel restriction sites allowed directional cloning of the vanRG gene under the control of the inducible Pspank promoter and upstream from PUG and the cat gene of pAT952 (pDR111ΩPspanktermPUGcat).

pAT956(pDR111ΩPspankvanURGcat), pAT958(pDR111ΩPspankvanRSGcat), pAT960(pDR111ΩPspankvanURSGcat) pAT961(pDR111ΩPspankvanRSGcat) and pAT962(pDR111ΩPspankvanURSGcat). The vanURG, vanRSG, and vanURSG genes of BM4518 were amplified using primer pairs UGNH2-RGCOOH, RGNH2-SGCOOH, and
UG\textsubscript{NH2}-SG\textsubscript{COOH} (S2 Table), respectively, and BM4518 total DNA as a template. Oligodeoxynucleotides UG\textsubscript{NH2} and RG\textsubscript{NH2} harbored a HindIII restriction site and 21 bases complementary to the sequence upstream from vanU\textsubscript{G} or 17 bases complementary to the sequence upstream from vanR\textsubscript{G}. Primers RG\textsubscript{COOH} and SG\textsubscript{COOH} contained each Sall and Nhel restriction sites, the stop codon and 14 or 13 bases complementary to the 3' end of respectively vanR\textsubscript{G} and vanS\textsubscript{G} of BM4518. The HindIII and Sall restriction sites allowed directional cloning of vanUR\textsubscript{G}, vanRS\textsubscript{G}, and vanURSG upstream from the cat reporter gene of shuttle vector pAT949 (pDR111\(\Omega\textsubscript{spank}^{\text{cat}}\)) carrying the inducible \(\text{P}_{\text{spank}}\) promoter to generate pAT955 (pDR111\(\Omega\text{spank}^{\text{vanURG}}\text{P}_{\text{UG}}\text{Gcat}^{\text{cat}}\)), pAT957 (pDR111\(\Omega\text{spank}^{\text{vanRSG}}\text{P}_{\text{UG}}\text{Gcat}^{\text{cat}}\)), and pAT959 (pDR111\(\Omega\text{spank}^{\text{vanURSG}}\text{P}_{\text{UG}}\text{Gcat}^{\text{cat}}\)). The 183-bp Nhel-Sall fragment carrying the \(P_{\text{UG}}\) promoter obtained above by amplification was cloned in pAT955 (pDR111\(\Omega\text{spank}^{\text{vanURG}}\text{P}_{\text{UG}}\text{Gcat}^{\text{cat}}\)), pAT957 (pDR111\(\Omega\text{spank}^{\text{vanRSG}}\text{P}_{\text{UG}}\text{Gcat}^{\text{cat}}\)), and pAT959 (pDR111\(\Omega\text{spank}^{\text{vanURSG}}\text{P}_{\text{UG}}\text{Gcat}^{\text{cat}}\)) digested with the same enzymes to generate pAT956 (pDR111\(\Omega\text{spank}^{\text{vanURG}}\text{P}_{\text{UG}}\text{Gcat}^{\text{cat}}\)), pAT958 (pDR111\(\Omega\text{spank}^{\text{vanRSG}}\text{P}_{\text{UG}}\text{Gcat}^{\text{cat}}\)), and pAT960 (pDR111\(\Omega\text{spank}^{\text{vanURSG}}\text{P}_{\text{UG}}\text{Gcat}^{\text{cat}}\)). The 177-bp Nhel-Sall fragment carrying the \(P_{\text{UG}}\) resistance promoter amplified by PCR from BM4518 DNA with primers PYG1 and PYG2 (S2 Table) was cloned in pAT957 (pDR111\(\Omega\text{spank}^{\text{vanRSG}}\text{P}_{\text{UG}}\text{Gcat}^{\text{cat}}\)), and pAT959 (pDR111\(\Omega\text{spank}^{\text{vanURSG}}\text{P}_{\text{UG}}\text{Gcat}^{\text{cat}}\)) digested with the same enzymes to generate, respectively, pAT961 (pDR111\(\Omega\text{spank}^{\text{vanRSG}}\text{B}_{\text{YG}}\text{cat}^{\text{cat}}\)), and pAT962 (pDR111\(\Omega\text{spank}^{\text{vanURSG}}\text{B}_{\text{YG}}\text{cat}^{\text{cat}}\)).

pAT964 (pDR111\(\Omega\text{spank}^{\text{vanUGterm}}\text{P}_{\text{UG}}\text{Gcat}^{\text{cat}}\)), pAT965 (pDR111\(\Omega\text{spank}^{\text{vanUGterm}}\text{P}_{\text{UG}}\text{Gcat}^{\text{cat}}\)), pAT966 (pDR111\(\Omega\text{spank}^{\text{vanRUGterm}}\text{P}_{\text{UG}}\text{Gcat}^{\text{cat}}\)), pAT967 (pDR111\(\Omega\text{spank}^{\text{vanURUGterm}}\text{P}_{\text{UG}}\text{Gcat}^{\text{cat}}\)), pAT968 (pDR111\(\Omega\text{spank}^{\text{vanURSGterm}}\text{P}_{\text{UG}}\text{Gcat}^{\text{cat}}\)), pAT969 (pDR111\(\Omega\text{spank}^{\text{vanURSGterm}}\text{P}_{\text{UG}}\text{Gcat}^{\text{cat}}\)), pAT970 (pDR111\(\Omega\text{spank}^{\text{vanURSGterm}}\text{P}_{\text{YG}}\text{Gcat}^{\text{cat}}\)), and pAT971 (pDR111\(\Omega\text{spank}^{\text{vanURSGterm}}\text{P}_{\text{YG}}\text{Gcat}^{\text{cat}}\)). The Nhel terminator fragment amplified by PCR with oligodeoxynucleotides T4F-Nhel and T4R-Nhel/KpnI (S2 Table) was cloned, respectively, in pAT951 (pDR111\(\Omega\text{spank}^{\text{vanUG}}\text{cat}^{\text{cat}}\)), pAT953 (pDR111\(\Omega\text{spank}^{\text{vanUG}}\text{cat}^{\text{cat}}\)), pAT954 (pDR111\(\Omega\text{spank}^{\text{vanRUG}}\text{cat}^{\text{cat}}\)), pAT956 (pDR111\(\Omega\text{spank}^{\text{vanURUG}}\text{cat}^{\text{cat}}\)), pAT960 (pDR111\(\Omega\text{spank}^{\text{vanURSG}}\text{cat}^{\text{cat}}\)), pAT958 (pDR111\(\Omega\text{spank}^{\text{vanURSG}}\text{cat}^{\text{cat}}\)), pAT962 (pDR111\(\Omega\text{spank}^{\text{vanURSG}}\text{cat}^{\text{cat}}\)) and pAT961 (pDR111\(\Omega\text{spank}^{\text{vanURSG}}\text{cat}^{\text{cat}}\)) digested with Nhel.

pAT972 (pDR111\(\Omega\text{spank}^{\text{vanUSGterm}}\text{cat}^{\text{cat}}\)). The 1,144-bp fragment containing the \(\text{vanS}_{\text{G}}\) gene of BM4518 was amplified using primer pair \(\text{SG}\text{NH2-SG}\text{COOH}^{\text{cat}}\) (S2 Table) and total DNA of the corresponding strain as a template. The Nhel and Sall restriction sites allowed directional cloning of \(\text{vanS}_{\text{G}}\) downstream from the \(\text{vanU}_{\text{G}}\) gene and upstream from the \(\text{cat}\) gene of pAT951 (pDR111\(\Omega\text{spank}^{\text{vanUG}}\text{cat}^{\text{cat}}\)) to generate pAT963 (pDR111\(\Omega\text{spank}^{\text{vanUSG}}\text{cat}^{\text{cat}}\)).

The EcoRI fragment harboring the \(\text{vanUS}_{\text{G}}\) genes from pAT963 (pDR111\(\Omega\text{spank}^{\text{vanUSG}}\text{cat}^{\text{cat}}\)) was replaced by the EcoRI fragment carrying the \(\text{vanRS}_{\text{G}}\) genes of pAT971 (pDR111\(\Omega\text{spank}^{\text{vanRSG}}\text{termP}_{\text{YG}}\text{Gcat}^{\text{cat}}\)) to generate pAT972 (pDR111\(\Omega\text{spank}^{\text{vanUSG}}\text{termcat}^{\text{cat}}\)).

Construction of strains
Plasmids pDR111, pAT949, pAT950, pAT952, pAT964, pAT965, pAT966, pAT967, pAT968, pAT969, pAT970, pAT971, and pAT972 were introduced by transformation into vancomycin susceptible \(E.\text{coli}\) NR698 and transformants were selected on agar containing chloramphenicol (10 g/ml) or ampicillin (100 μg/ml, for pDR111) (Tables 1 and 2).

In Gram-positive bacteria, pGhost9 [36] which replicates at 28°C but is lost above 37°C, allowed construction of \(E.\text{faecalis}\) BM4522 derivatives by insertion inactivation. Plasmids pAT945 (pGhost9\(\text{Cm}\Delta \text{vanUG}_{\text{G}}\)), pAT946 (pGhost9\(\text{Cm}\Delta \text{vanR}_{\text{G}}\)), and pAT947 (pGhost9\(\text{Cm}\Delta \text{vanS}_{\text{G}}\)) were electrotransformed into \(E.\text{faecalis}\) BM4522 [11] to generate, respectively, BM4720(Δ\text{vanUG}_{\text{G}}), BM4721(ΔvanR_{\text{G}}), and BM4722(ΔvanS_{\text{G}}) (S1 Table). Plasmid pAT973
(pGhost9CmΔvanR’G) was electrot transformed into *E. faecalis* BM4721(ΔvanR’G) to generate the double mutant BM4723(ΔvanR’G, ΔvanR’G). Transformants were selected at the permissive temperature (28°C) on M17 plates containing 10g/ml of chloramphenicol and 0.5% glucose. A colony of each transformant was inoculated into 50 ml of M17 broth containing 0.5% glucose and incubated for 2h at 28°C. The culture was then shifted to a non-permissive temperature (42°C) for 2 h and integrants, following a first recombination event, were selected at 42°C on M17 agar containing chloramphenicol (10g/ml). Plasmid excision, by a second recombination event, was favored by subculturing at 28°C in the absence of chloramphenicol and plasmid loss was screened for by plating at 42°C on M17-glucose followed by replica plating on chloramphenicol. The integration locus was determined by PCR following digestion with SmaI and sequencing.

**Enzyme assays**

For preparation of extracts, 8 ml of an overnight culture were added to 100 ml of broth in the absence or in the presence of vancomycin and strains were grown until OD_{600} = 0.8 in 250 ml bottles with shaking at 110 rpm. The cells were harvested by centrifugation, washed in 0.1M phosphate buffer pH 7.0, resuspended in the same buffer, lysed by sonication, followed by centrifugation at 10,000 g during 45 min. The resuspended pellet for VanTG racemase [11] and supernatant for CAT activity, were assayed as described [38].

**Genome sequencing, assemblies and annotation**

Total DNA from BM4518 and BM4522 strains was purified and sequencing library preparation was carried out using the Nextera DNA Sample Preparation kit (Illumina, San Diego, CA), according to manufacturer’s specifications. Quality and quantity of each sample library was measured on an Agilent Technologies 2100 Bioanalyzer (Santa Clara, CA). Libraries were normalized to 2nM, multiplexed and subjected to 250-bp paired end sequencing (Illumina MiSeq). On average, 5 million high-quality paired-end reads were collected for each strain, representing >220-fold coverage of the ~2.9 Mb genomes. Reads were assembled de novo utilizing CLC Genomics Workbench (CLC bio, Cambridge, MA). Functional annotations were performed using a custom pipeline as described previously [39].

**Determination of growth rates**

Growth rates were determined in microplates coupled to a spectrophotometer iEMS reader (Labsystems). Strains were grown overnight at 37°C without or with 1 μg/ml of vancomycin. The cultures were diluted at OD 0.15 into 10 ml of broth without or with vancomycin (1μg/ml) and grown at 37°C with shaking until the beginning of the stationary phase. The cultures were diluted 1/1,000 to inoculate 10^5 bacteria into 200 μl of broth in a 96-well microplate that was incubated overnight at 37°C with shaking. Absorbance was measured at 600 nm every 3 min. Each culture was replicated three times in the same microplate. Growth rates performed in three independent experiments were determined at the beginning of the exponential phase and the relative growth rates were calculated as the ratio of the growth rate of the strain induced by vancomycin versus that of the non induced strain.

**Supporting Information**

S1 Fig. Effect of mutations in the PUG promoter regulatory region on the in vitro binding of VanUG. (A) Sequence of the wild-type (WT) and mutated promoter regions. The two 14-bp imperfect inverted repeats corresponding to the putative binding sites are indicated in orange and pink and by arrows. A DNA fragment (197 bp) was obtained with PUG3 plus labeled...
VanG126 and mutated PUG5 plus labeled VanG126 primers (S2 Table) leading to the WT and corresponding mutated (mutant 1) promoter region, respectively. A DNA fragment (293 bp) was obtained with labeled VanG12 plus PUG4 and labeled VanG12 plus mutated PUG6 primers (S2 Table) leading to the WT and corresponding mutated (mutant 2) promoter region, respectively. Numbering relative to the transcription start site is indicated above the sequences. Only bases differing from the WT sequence are shown in the mutated fragments. (B) Gel shift analysis. The labeled fragments corresponding to the WT and mutated (mutant 1 and mutant 2) promoter regions were incubated in the absence or in the presence of decreasing concentrations of purified VanU_G protein indicated above the lanes.

S2 Fig. Binding of δ70 RNA polymerase of *E. coli* to (A) the PUG regulatory and (B) PYG resistance promoters by DNase I footprinting analysis. (A) A 357-bp DNA fragment was amplified from the *P*<sub>UG</sub> promoter region using a labeled reverse primer (VanG126) (S2 Table) to radiolabel the template strand and the DNA probe was incubated without and with δ70 RNA polymerase at 50 nM. (B) A 233-bp DNA fragment was amplified from the *P*<sub>YG</sub> promoter region using a labeled reverse primer (YG10) (S2 Table) to radiolabel the template strand and the DNA probe was analysed similarly. The brackets indicate the regions protected from DNase I cleavage by δ70 RNA polymerase, and the co-ordinates of protection relative to the transcriptional start site are indicated on the right. M is the A+G Maxam and Gilbert sequencing reaction lane of the probes used as a size marker and the nucleotide positions are indicated at the left. RNAP, RNA polymerase.

S3 Fig. Comparison of the deduced amino acid sequences of VanR<sub>G</sub> with VanR<sub>G</sub>' (A) and of VanU<sub>G</sub> from *E. faecalis* BM4518 with Cro/C1<sub>cd</sub> from *Clostridium difficile* (77% identity, GenBank N° EQJ96019) and Cro/C1<sub>bf</sub> from *Butyvibrio fibrisolvens* (52% identity, GenBank N° WP_022757627) (B). Identical amino acids are indicated by dashes below the alignment.

S4 Fig. Competition between VanR<sub>G</sub> and VanU<sub>G</sub> for binding to the PYG resistance promoter by DNase I footprinting. A 233-bp DNA fragment was amplified from the *P*<sub>YG</sub> region using a labeled reverse primer (YG10) (S2 Table) to radiolabel the template strand. Increasing amounts of VanU<sub>G</sub> and two fixed amounts of VanR<sub>G</sub> indicated at the top were incubated with the DNA probe. The bracket indicates the region protected from DNase I cleavage by VanR<sub>G</sub> and/or VanU<sub>G</sub> and the co-ordinates of protection relative to the transcriptional start site are indicated on the left. M is the A+G Maxam and Gilbert sequencing reaction lane of the probes used as a size marker and the nucleotide positions are indicated at the right.

S1 Table. Bacterial strains and plasmids.

S2 Table. Oligonucleotide primers used.

Acknowledgments

We thank F. Lebreton for whole genome sequencing of strains BM4518 and BM4522, D. Rudner for the gift of plasmid pDR111, T.Silhavy for providing *E. coli* NR698, A.Kolb, and T. Msa-dek for helpful discussions.
Author Contributions
Conceived and designed the experiments: FD VM PC. Performed the experiments: FD. Analyzed the data: FD VM PC. Contributed reagents/materials/analysis tools: PC. Wrote the paper: FD VM PC.

References
1. Arias CA, Murray BE (2012) The rise of the Enterococcus: beyond vancomycin resistance. Nat Rev Microbiol 10: 266–278. doi: 10.1038/nrmicro2761 PMID: 22421879
2. Reynolds PE (1989) Structure, biochemistry and mechanism of action of glycopeptide antibiotics. Eur J Clin Microbiol Infect Dis 8: 943–950. PMID: 2532132
3. Depardieu F, Podgajen I, Leclercq R, Collatz E, Courvalin P (2007) Modes and modulations of antibiotic resistance gene expression. Clin Microbiol Rev 20: 79–114. PMID: 17223624
4. Boyd DA, Willey BM, Fawcett D, Gillani N, Mulvey MR (2008) Molecular characterization of Enterococcal N06-0364 with low-level vancomycin resistance harboring a novel D-Ala-D-Ser gene cluster, vanL. Antimicrob Agents Chemother 52: 2667–2672. doi: 10.1128/AAC.01516-07 PMID: 18458129
5. Lebreton F, Depardieu F, Bourdon N, Fines-Guyon M, Berger P, Camiade S, et al. (2011) D-Ala-D-Ser VanN-type transferable vancomycin resistance in Enterococcus faecium. Antimicrob Agents Chemother 55: 4606–4612. doi: 10.1128/AAC.00714-11 PMID: 21807981
6. Xu X, Lin D, Yan G, Ye X, Wu S, Guo Y, et al. (2010) vanM, a new glycopeptide resistance gene cluster found in Enterococcus faecium. Antimicrob Agents Chemother 54: 4643–4647. doi: 10.1128/AAC.01710-09 PMID: 20733041
7. Depardieu F, Courvalin P, Msadek T (2003) A six amino acid deletion, partially overlapping the VanSB G2 ATP-binding motif, leads to constitutive glycopeptide resistance in VanB-type Enterococcus faecium. Mol Microbiol 50: 1069–1083. PMID: 14617162
8. Depardieu F, Bonora MG, Reynolds PE, Courvalin P (2003) The vanG glycopeptide resistance operon from Enterococcus faecalis revisited. Mol Microbiol 50: 931–948. PMID: 14617152
9. Wright GD, Holman TR, Walsh CT (1993) Purification and characterization of VanR and the cytosolic domain of VanS: a two-component regulatory system required for vancomycin resistance in Enterococcus faecium BM4147. Biochemistry 32: 5057–5063. PMID: 8494882
10. Arthur M, Depardieu F, Gerbaud G, Galimand M, Leclercq R, Courvalin P (2011) D-Ala-D-Ser VanN-type transferable vancomycin resistance in Enterococcus faecium. Antimicrob Agents Chemother 55: 4606–4612. doi: 10.1128/AAC.00714-11 PMID: 21807981
11. Ruiz N, Falcone B, Kahne D, Silhavy TJ (2005) Chemical conditionality: a genetic strategy to probe organelle assembly. Cell 121: 307–317. PMID: 15851036
12. Holman TR, Wu Z, Wanner BL, Walsh CT (1994) Identification of the DNA-binding site for the phosphorylated VanR protein required for vancomycin resistance in Enterococcus faecium. Biochemistry 33: 4625–4631. PMID: 8161518
13. Koudelka GB, Lam CY (1993) Differential recognition of OR1 and OR3 by bacteriophage 434 repressor and Cro. J Biol Chem 268: 3812–3817. PMID: 8226917
14. Cervin MA, Lewis RJ, Brannigan JA, Spiegelman GB (1998) The Bacillus subtilis regulator SinR inhibits spoIIG promoter transcription in vitro without displacing RNA polymerase. Nucleic Acids Res 26: 3806–3812. PMID: 9685500
15. Newman JA, Rodrigues C, Lewis RJ (2013) Molecular basis of the activity of SinR protein, the master regulator of biofilm formation in Bacillus subtilis. J Biol Chem 288: 10776–10787. doi: 10.1074/jbc.M113.455992 PMID: 23430750
16. Makino K, Shinagawa H, Amemura M, Kawamoto T, Yamada M, Nakata A. (1989) Signal transduction in the phosphate regulon of Escherichia coli involves phosphotransfer between PhoR and PhoB proteins. J Mol Biol 210: 551–558. PMID: 2693738
17. Ansaldi M, Simon G, Lepelletier M, Mejean V (2000) The TorR high-affinity binding site plays a key role in both torR autoregulation and torCAD operon expression in Escherichia coli. J Bacteriol 182: 961–966. PMID: 10649219
18. Igo MM, Ninfa AJ, Stock JB, Silhavy TJ (1989) Phosphorylation and dephosphorylation of a bacterial transcriptional activator by a transmembrane receptor. Genes Dev 3: 1725–1734. PMID: 2558046
20. Kamberov ES, Atkinson MR, Chandran P, Ninfa AJ (1994) Effect of mutations in *Escherichia coli* gltL (ntrB), encoding nitrogen regulator II (NRII or NtrB), on the phosphatase activity involved in bacterial nitrogen regulation. J Biol Chem 269: 28294–28299. PMID: 7961767

21. Arthur M, Depardieu F, Courvalin P (1999) Regulated interactions between partner and non partner sensors and responses regulators that control glycopeptide resistance gene expression in enterococci. Microbiology 145: 1849–1858. PMID: 10463151

22. Ogura M, Yoshikawa H, Chibazakura T (2014) Regulation of the response regulator gene degU through the binding of SinR/SlrR and exclusion of SinR/SlrR by DegU in *Bacillus subtilis*. J Bacteriol 196: 873–881. doi: 10.1128/JB.01321-13 PMID: 23139400

23. Mittal M, Pechter KB, Picossi S, Kim HJ, Kerstein KO, Sonenshein AL (2013) Dual role of CcpC protein in regulation of aconitase gene expression in *Bacillus subtilis*. J Bacteriol 196: 873–881. doi: 10.1128/JB.01321-13 PMID: 23139400

24. Buelow DR, Raivio TL (2010) Three (and more) component regulatory systems—auxiliary regulators of bacterial histidine kinases. Mol Microbiol 75: 547–566. doi: 10.1111/j.1365-2958.2009.06982.x PMID: 19943903

25. Foucault ML, Depardieu F, Courvalin P, Grillot-Courvalin C (2010) Inducible expression eliminates the fitness cost of vancomycin resistance in enterococci. Proc Natl Acad Sci USA 107: 16964–16969. doi: 10.1073/pnas.1006855107 PMID: 20833818

26. Studier FW, Moffatt BA (1986) Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J Mol Biol 189: 113–130. PMID: 3573035

27. Maxam AM, Gilbert W (1977) A new method for sequencing DNA. Proc Natl Acad Sci U S A 74: 560–564. PMID: 3573035

28. Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254. PMID: 492051

29. Chastanet A, Fert J, Msadek T (2003) Comparative genomics reveal novel heat shock regulatory mechanisms in *Staphylococcus aureus* and other Gram-positive bacteria. Mol Microbiol 47: 1061–1073. PMID: 12581359

30. Lebreton F, van Schaik W, McGuire AM, Godfrey P, Griggs A, Mazumdar V (2013) Emergence of epidemic multidrug-resistant Enterococcus faecium from animal and commensal strains. MBio 4.