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Inducible expression eliminates the fitness cost of vancomycin resistance in enterococci

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Inducible vancomycin resistance in enterococci is due to a sophisticated mechanism that combines synthesis of cell wall peptidoglycan precursors with low affinity for glycopeptides and elimination of the normal target precursors. Although this dual mechanism, which involves seven genes organized in two operons, is predicted to have a high fitness cost, resistant enterococci have disseminated worldwide. We have evaluated the biological cost of VanB-type resistance due to acquisition of conjugative transposon Tn*1549* in *Enterococcus faecium* and *Enterococcus faecalis*. Because fitness was dependent on the integration site of Tn*1549*, an isogenic set of *E. faecalis* was constructed to determine the cost of inducible or constitutive expression of resistance or of carriage of Tn*1549*. A luciferase gene was inserted in the integrase gene of the transposon to allow differential quantification of the strains in cocultures and in the digestive tract of gnotobiotic mice. Both in vitro and in vivo, carriage of inactivated or inducible Tn*1549* had no cost for the host in the absence of induction by vancomycin. In contrast, induced or constitutively resistant strains not only had reduced fitness but were severely impaired in colonization ability and dissemination among mice. These data indicate that tight regulation of resistance expression drastically reduces the biological cost associated with vancomycin resistance in *Enterococcus* spp. and accounts for the widespread dissemination of these strains. Our findings are in agreement with the observation that regulation of expression is common in horizontally acquired resistance and represents an efficient evolutionary pathway for resistance determinants to become selectively neutral.

glycopeptide resistance | biological cost | *Enterococcus* | transposon | two component regulatory system

One of the key parameters influencing emergence and stability of antibiotic resistance is the biological cost that resistance determinants impose on cell growth (1). Many studies have shown that resistant bacteria are less fit than their susceptible counterpart, although they can acquire compensatory mutations that restore fitness (2). However, most reports have dealt with resistance due to chromosomal mutations, and fewer have evaluated the fitness change due to acquisition of mobile genetic elements conferring clinically relevant resistance (3).

Enterococci are commensals of the intestine of humans and animals but can also be opportunistic pathogens. This bacterial genus has acquired resistance to many antibiotics, and glycopeptides are often the ultimate treatment of infections due to these Gram-positive cocci. This class of drugs inhibits peptidoglycan synthesis by binding to the C-terminal D-alanyl-D-alanine (D-Ala-D-Ala) of pentapeptide precursors, preventing transglycosylation and transpeptidation in cell wall assembly (4).

Acquired resistance to the glycopeptides vancomycin and teicoplanin in enterococci is due to the synthesis of modified peptidoglycan precursors ending in D-alanyl-D-lactate (D-Ala-D-Lac), to which glycopeptides exhibit low binding affinities, together with elimination of the high-affinity D-Ala-D-Ala ending precursors (5, 6). In VanB-type resistant strains, similar to VanA-type strains, synthesis of D-Ala-D-Lac requires a dehydrogenase ($VanH_B$) that converts pyruvate to D-Lac and a ligase (VanB) of altered specificity compared with the host D-Ala:D-Ala ligase (Ddl). Removal of precursors terminating in D-Ala is mediated by a D,D-dipeptidase ($VanX_B$) and a D,D-carboxypeptidase ($VanY_B$) (7). Expression of resistance is induced by vancomycin and regulated by a two-component regulatory system composed of a sensor ($VanS_B$) and a regulator ($VanR_B$) that acts as a transcriptional activator (8). Induction of the sensor leads to expression of the regulatory ($vanR_BS_B$) and resistance ($vanH_BBX_B$) operons (6). Mutations in $vanS_B$ leading to constitutive expression of resistance have been obtained in vitro and in vivo but are rare in clinical settings (8).

The *vanA* operon is part of transposon Tn*1546*, which is often carried by self-transferable plasmids, accounting for its spread (9). Dissemination of VanB-type resistance results from the spread of conjugative transposon Tn*1549* (10) located on plasmids or in the chromosome. Despite the sophisticated dual mechanism of resistance, glycopeptide-resistant enterococci have disseminated worldwide.

Two studies have investigated the burden of glycopeptide resistance imposed on enterococci, with conflicting results. A fitness disadvantage was reported in VanA-type *Enterococcus faecium* by comparing the in vitro competitiveness of the resistant strain harboring a >100-kb *vanA* plasmid with that of its plasmid-free counterpart (11). However, the 4% reduction in fitness could be due to the cost of carrying a large-size plasmid. In another study, the growth rates of VanB-type *E. faecium* clinical isolates were found to be similar to those of nonisogenic susceptible strains (12). Neither of these works addressed the cost of vancomycin resistance adequately because isogenic enterococci differing only in resistance were not studied.

We have taken advantage of the availability of VanB-type enterococci transconjugants to evaluate the cost of vancomycin resistance. Isogenic strains have been constructed to determine the fitness burden due to inducible or constitutive resistance or to carriage of transposon Tn*1549*. Using bioluminescence imaging and a mouse model, we have demonstrated in vitro and in vivo that inactivated or inducible Tn*1549* is not costly for the host in the absence of vancomycin, whereas induced or constitutive expression of the *vanB* operon not only confers a high fitness burden to the bacteria but also modulates colonization ability and transmission between mammalian hosts.

Results

Impact of Vancomycin Resistance on Fitness of Enterococci in Vitro. ***Fitness of E. faecium depends on Tn1549 integration site.*** Nineteen *E. faecium* 64/3 transconjugants, in which Tn*1549* (Fig. 1) had inserted at a single copy in 16 sites of the chromosome, were obtained in vivo or in vitro (13). The growth rates of the trans-

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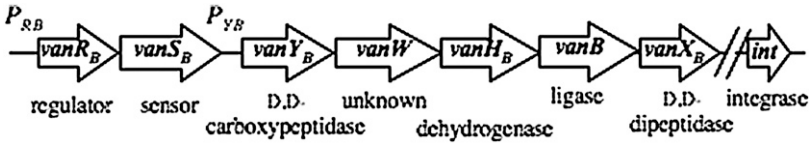

Fig. 1. Schematic representation of part of Tn*1549*. Open arrows represent coding sequences and indicate direction of transcription.

conjugants were determined in monocultures and compared with that of the 64/3 recipient (Fig. 2) (14). Among the 17 in vitro transconjugants, 14 had growth rates similar to that of 64/3, 2 strains (T25 and T28) as well as the 2 in vivo transconjugants (T2 and T3) had a significantly reduced relative growth rate, and 1 (T12) had a significantly, albeit moderately, higher relative growth rate. Integration of Tn*1549* in a coding sequence was not costly in seven of nine transconjugants. For the four transconjugants with a reduced growth rate, integration occurred in noncoding (T2 and T25) or coding (T3 and T28) sequences. These results indicate that the integration site of a genetic element can have a neutral, positive, or negative impact on host fitness and reinforce the requirement for isogenic strains for the study of the biological cost of resistance.

***Tn1549* carriage does not alter fitness.** Isogenic *Enterococcus faecalis* JH2-2 (15) derivatives in which resistance to vancomycin is inducible, constitutive, or has been inactivated were constructed (Table 1 and Fig. S1). In inducible *E. faecalis* JH2-2::I, Tn*1549* (Fig. 1) had been inserted in the chromosomal gene for a potassium transporter of the Trk family (accession no. ZP-03948272). A single copy of the transposon was present as determined by pulsed-field gel electrophoresis and hybridization with a *vanB*-specific probe.

The exponential growth rate of JH2-2::I was higher than that of JH2-2 (Table 1), with a ratio significantly greater than 1 (1.22 ± 0.07; $P < 0.01$). For more sensitive fitness evaluation, JH2-2::I was mixed with JH2-2 at an initial ratio of 1:1, and the mixture was grown in brain heart infusion (BHI) medium for *ca.* 50 generations. The inducible strain was as competitive as JH2-2 (relative fitness, 0.98), confirming the absence of fitness burden of Tn*1549* carriage.

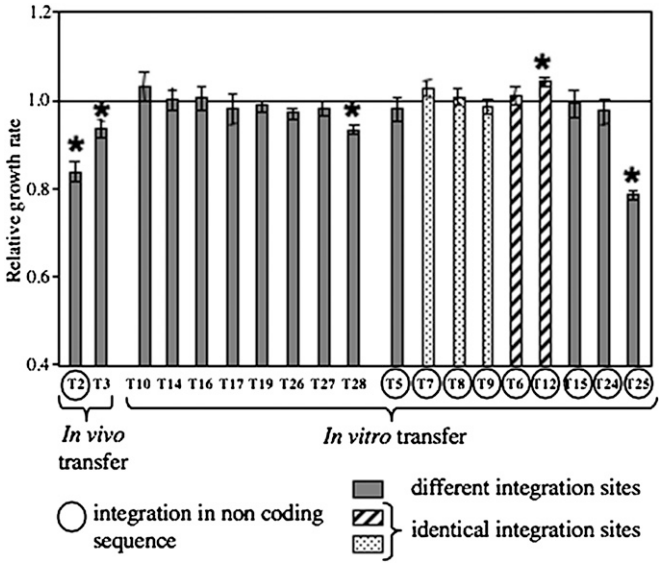

Fig. 2. Relative growth rates of *E. faecium* 64/3 transconjugants. Growth rates were determined at the beginning of the exponential phase. Relative growth rate represents the ratio of growth of each transconjugant to that of recipient 64/3 (taken as 1). The values are the means of two independent cultures from at least three separate experiments with error deviations. *Significant difference ($P < 0.05$) of the mean value.

***Noninduced* VanB-type resistance is not costly for the host.** Susceptible JH2-2::S derived from JH2-2::I (Fig. S1*B*) by introducing two point mutations in $vanX_B$ had background levels of D,D-dipeptidase activity (Fig. S2). The growth rate of both strains was indistinguishable (Table 1), demonstrating that there is no fitness cost associated with the noninduced transposon. This was confirmed in competition experiments, in which no difference in fitness between the two strains was detected (Table 2).

***Expression of* VanB-type resistance reduces fitness significantly.** To induce expression of resistance genes, JH2-2:I was grown in the presence of a subinhibitory concentration of vancomycin [8 μg/mL; i.e., 1/32 of the minimum inhibitory concentration (MIC)]. The growth rate was significantly reduced compared with that of JH2-2::S (0.52 vs. 0.97), indicating that expression of resistance was highly costly for the host. A similar reduction was observed upon induction in clinical isolates (Table 1) *E. faecalis* TJ282 (0.61 vs. 1) and *E. faecium* BM4524 (0.78 vs. 1).

Constitutive resistance in VanB-type isolates is due to substitutions in $VanS_B$ near histidine 233 of the putative autophosphorylation site (6) or to a 6-aa deletion overlapping the G2 ATP-binding domain (8). Analysis of the $vanS_B$ sequence in three constitutive mutants of JH2-2::I selected on teicoplanin revealed the 18-bp deletion corresponding to the same 6-aa deletion in JH2-2::C1 and point mutations leading to substitutions $P_{238}T$ and $S_{232}Y$ near H_{233} in JH2-2::C2 and -::C3, respectively. Constitutivity was confirmed by detection of $VanX_B$ activity (Fig. S2*A*) and of pentadepsipeptide in the mutants in the absence of induction (Fig. S2*B*). In monoculture without vancomycin, JH2-2::C1, -::C2, and -::C3 displayed significantly reduced growth rates compared with JH2-2::S (Table 1), with ratios significantly less than 1 (0.78, 0.53, and 0.71, respectively). In competition experiments, a disadvantage was observed for constitutive mutants JH2-2::C1 (0.90 ± 0.03; Table 2), -::C2 (0.80), and -::C3 (0.81) relative to JH2-2::S. Taken together these results demonstrate that constitutive resistance leads to a significant fitness reduction of the host in the absence of vancomycin.

Exclusive use of the altered peptidoglycan biosynthetic pathway is very costly. Enterococci with a mutated *ddl* that are constitutively resistant after mutations in $vanS_B$ have been isolated in clinical settings (8) and in vitro (6). These strains rely entirely on the resistance pathway for peptidoglycan synthesis and produce exclusively precursors ending in D-Ala-D-Lac. Two such double mutants, TJ282::C4 and TJ282::C5 (Table 1) (16), had growth rate ratios significantly less than 1 relative to the inducibly resistant parent *E. faecalis* TJ282 (0.77 and 0.75, respectively). Similarly, BM4524::C6 (8) with the same 6-aa deletion as that in *E. faecalis* JH2-2::C1 had a reduced growth rate (0.65) and a competitive disadvantage (Table 2) relative to the parental strain BM4524. These results indicate that exclusive synthesis of modified cell wall is highly costly for the host or that abnormal cell wall impairs growth.

Evaluation of Fitness in Vivo. The clinical relevance of the impact of resistance on fitness are best studied in vivo in the usual environment of the bacteria. Because enterococci are commensals of the digestive tract, the gnotobiotic mouse model is particularly suited to monitor the fate of isogenic strains, alone or in competition, in a controlled intestinal ecosystem. We have shown that bioluminescence imaging is most convenient to study intestinal colonization of mice by *Escherichia coli* (17). A good correlation

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Table 1. Glycopeptide resistance and growth rates

Strain	Relevant properties	MIC* Vm	MIC* Te	Growth rate (min^{-1})†
E. faecalis				
JH2-2	Wild-type strain, Fus^R Rif^R	1	1	0.017 ± 0.003
JH2-2::I	JH2-2::Tn*1549*	256	1	0.020 ± 0.004
JH2-2::IΩ*lucR*	Derivative of JH2-2::I producing luciferase	256	1	0.020 ± 0.002
JH2-2::S	Susceptible derivative of JH2-2::I	4	0.5	0.021 ± 0.001
JH2-2::SΩ*lucR*	Derivative of JH2-2::S producing luciferase	4	0.5	0.021 ± 0.001
JH2-2::C1	Constitutive derivative of JH2-2::I	512	4	0.018 ± 0.002
JH2-2::C1Ω*lucR*	Derivative of JH2-2::C1 producing luciférase	512	4	0.018 ± 0.002
JH2-2::C2	Constitutive derivative of JH2-2::I	256	2	0.011 ± 0.005
JH2-2::C3	Constitutive derivative of JH2-2::I	256	64	0.015 ± 0.002
TJ282	Clinical isolate harboring Tn*1549*	16	1	0.028 ± 0.000
TJ282 C4	Constitutive derivative of TJ282	1,024	256	0.021 ± 0.001
TJ282 C5	Constitutive derivative of TJ282	1,024	256	0.021 ± 0.000
E. faecium				
BM4524	Clinical isolate harboring Tn*1549*	512	2	0.028 ± 0.003
BM4524 C6	Constitutive derivative of BM4524	1,024	4,096	0.018 ± 0.001

R, resistance; Vm, vancomycin; Te, teicoplanin.
*MICs in μg/mL were determined with 10^5 cfu per spot on BHI agar after 24 h of incubation at 37 °C.
†Exponential growth rate measured in the absence of antibiotic; average of at least four independent experiments ± SE.

between the cfu counts and the photons/s obtained from serial dilutions of feces from mice fed with *lucR*-tagged enterococci (Fig. S1) indicated that photon emission accurately reflects bacterial numbers between *ca.* 5.10^6 and 10^8 cfu ($R^2 = 0.99$; Fig. S3).

Tn1549 carriage does not alter colonization ability. *E. faecalis* JH2-2, JH2-2::S, and JH2-2::I or their bioluminescent derivatives were separately inoculated intragastrically to groups of five mice. The transconjugants exhibited colonization profiles similar to that of JH2-2 with an initiation stage (0–1 d after feeding), followed by a maintenance stage ranging from 9 to 10 Log_{10} cfu/g of feces for at least 10 d (Fig. 3*A*). Similar levels of gut colonization of gnotobiotic mice by *Enterococcus* spp. have been reported (18).

Expression of VanB-type resistance impairs colonization. Because the 18-pb deletion is stable and has been found in a clinical isolate (8), strain JH2-2::C1 was selected for in vivo studies, tagged with the *lucR* gene (Table 1 and Fig. S1), and inoculated in five mice. At maintenance, the level of JH2-2::C1 (9.5 Log_{10} cfu/g of feces) was significantly, albeit moderately, lower than that of the susceptible or noninduced isogenic strains (≥9.8 Log_{10}) (Fig. 3*A*). To assess the impact of induction of resistance on colonization ability, two groups of five mice were intragastrically inoculated with *E. faecalis* JH2-2::I. The drinking water of one group was supplemented with a subinhibitory (1/32 of MIC) concentration of vancomycin (8 μg/mL) before intragastric inoculation and throughout the experiment. In these mice, JH2-2::I colonization was delayed and did not reach the level of 8.7 Log_{10} cfu/g of feces until 3 d after inoculation (Fig. 3*B*).

Tn1549 carriage does not alter cocolonization ability. When *E. faecalis* JH2-2::IΩ*lucR* and susceptible JH2-2 were coinoculated to the mice, after an initial slower colonization profile by the inducible strain, both strains were maintained in the digestive tract at similar levels of *ca.* 9–10 Log_{10} cfu/g of feces (Fig. 3*C*). It thus seems that, whether in vitro or in vivo in mice, the noninduced transposon does not bear any cost to the bacterial host.

Susceptible strain alters colonization ability of isogenic constitutively resistant bacteria during cocolonization. The ability of JH2-2::C1 to colonize the digestive tract in competition with JH2-2::S was tested in mice inoculated intragastrically with equal numbers of the two strains, either one or the other tagged with *lucR*. Higher heterogeneity in the colonization levels by JH2-2::C1 was observed among the mice (Fig. 3*D*). During the initiation stage, the levels of this strain ranged from 2 to 8.5 Log_{10} cfu/g of feces at day 1 and were always inferior to those of JH2-2::S (9–10 Log_{10} cfu/g of feces). Subsequently, JH2-2::C1 rapidly diminished to reach background levels (3–4 Log_{10} cfu/g of feces) at day 7 (Fig. 3*D*). The competitive disadvantage of the constitutive derivative was therefore observed in vitro as well as in vivo.

Constitutive resistance decreases dissemination ability. The ability of JH2-2::S, JH2-2::I, and JH2-2::C1 to disseminate from mouse to mouse was tested using bioluminescence (Fig. 4). Mice initially

Table 2. Relative fitness of resistant vs. susceptible strains in in vitro competition experiments

Strain	Ratio at end of each transfer* 1	2	3	4	5	s†	Relative fitness‡	P§
JH2-2::I/JH2-2::S	0.98	0.97	0.96	0.98	0.96	−0.018	0.98 ± 0.01	0.080
JH2-2::C1/JH2-2::S	0.99	0.95	0.86	0.86	0.82	−0.104	0.90 ± 0.03	0.0002
BM4524 C6/BM4524	0.93	0.92	0.87	0.58	0.40	−0.142	0.86 ± 0.02	0.00001

*Values represent means of the Log_{10} (cfu) ratios of the resistant population vs. the susceptible population at the end of each transfer (*ca.* 10 generations). Each experiment was carried out in triplicate and performed four times independently.
†Selection coefficient.
‡Fitness relative to that of susceptible parental strain. Values are mean ± SE of at least three experiments.
§Statistical significance of difference in fitness relative to the susceptible strain, which was set to 1 (Student's *t* test, 99% confidence limit).

colonized with the susceptible or inducibly resistant strain were hosted at day 7 in the same cage, and the feces of each mouse were analyzed during the 6 following days. *E. faecalis* JH2-2::IΩ*lucR* was able to disseminate from mouse 2 colonized with this strain to mouse 1, stably colonized by JH2-2::S. The inducible strain was detectable in the feces of mouse 1 after 2 d of contact with mouse 2 and reached a colonization level of 9 Log_{10} cfu/g of feces after 4 d (Fig. 4*A*, *Left*). Similarly, JH2-2::S disseminated from mouse 1 to mouse 2 (colonized with JH2-2::IΩ*lucR*) as early as day 1 and progressively reached a colonization level of 8.5 Log_{10} cfu/g of feces after 6 d of contact with mouse 1 (Fig. 4*A*, *Right*). However, when mouse 4, colonized with JH2-2::C1Ω*lucR*, was hosted with mouse 3, colonized with JH2-2::S, the constitutive strain remained below the detection levels in the feces of mouse 3 throughout the 6-d cohabitation and therefore seemed unable to disseminate (Fig. 4*B*, *Left*). In contrast, JH2-2::S was detected in the feces of mouse 4 after 2 d and gradually disseminated from mouse 3 to mouse 4 to reach, at day 6, a colonization level of 8.7 Log_{10} cfu/g of feces (Fig. 4*B*, *Right*), demonstrating that the susceptible strain was able to disseminate in a JH2-2::C1Ω*lucR* colonized mouse. Similar results were obtained with mice initially colonized with other combinations of strains tagged or not with *lucR*.

Taken together, these results indicate that constitutive expression of resistance markedly decreased the dissemination ability of the bacteria. The noninduced resistant strain disseminated as efficiently as its susceptible counterpart, accounting for widespread dissemination in clinical settings.

Discussion

Resistance to glycopeptides in VanA- and VanB-type strains is associated with a sophisticated dual biochemical mechanism that requires seven genes organized in two operons and combines synthesis of modified peptidoglycan precursors with low affinity for the drugs and elimination of the high-affinity precursors produced by the bacteria (6). Despite this complex mechanism, predicted to have a high biological cost, vancomycin-resistant enterococci have disseminated worldwide and represent a major cause of nosocomial infections (19). Of greater significance, resistance has disseminated from *Enterococcus* spp. to methicillin-resistant *Staphylococcus aureus* (MRSA) (20), against which glycopeptides are among the drugs of last resort.

Few studies have evaluated the fitness cost associated with antibiotic resistance transposons. Acquisition of Tn*1* and Tn*7*, conferring resistance to ampicillin and trimethoprim, respectively, have no impact on the fitness of *E. coli* (21). In another study, 80% of Tn*10* insertional mutants presented a fitness cost that was related to the insertion locus rather than to expression of tetracycline resistance (22). In our study, of 19 *E. faecium* transconjugants carrying a single copy of Tn*1549* integrated at various chromosomal loci (13) in the 14 there was no impact on fitness, and only four had reduced fitness (Fig. 2). Surprisingly, of two transconjugants (T12 and T6) that inserted at the same site and in the same orientation, one had a higher growth rate than the recipient (Fig. 2). A similar observation has been reported for two derivatives carrying Tn*1* (21). Thus, the integration site of a mobile genetic element may have an impact on fitness of the host, a result that emphasizes the importance of studying isogenic strains.

In the present work, VanB-type resistance was conferred by a single copy of transposon Tn*1549* stably integrated in the chromosome, and several isogenic *E. faecalis* derivatives were constructed to allow separate evaluation of the biological cost due to the carriage of the inactivated element or to the inducible or constitutive expression of resistance (Table 1 and Fig. S1). The in vitro growth rate of inducibly resistant *E. faecalis* JH2-2::I was higher than that of JH2-2 (Table 1), but because the strains were equally competitive, this transconjugant was well suited to evaluate the cost of the resistance mechanism. In addition, this observation

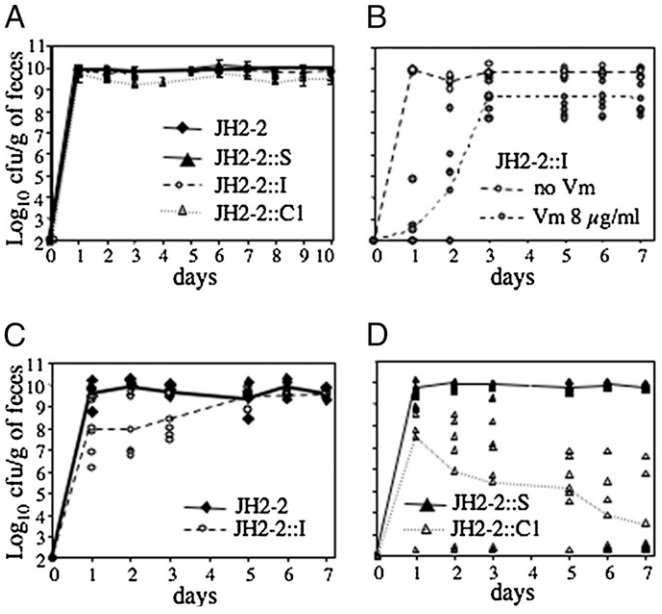

Fig. 3. Colonization (*Upper*) and cocolonization (*Lower*) of gnotobiotic mice. (*A*) 10^8 cfu of *E. faecalis* JH2-2, JH2-2::S, JH2-2::I, and JH2-2::C1 were separately inoculated intragastrically to groups of five germ-free mice. The mean Log_{10} cfu/g of feces of five mice was plotted against days of colonization. (*B*) 10^8 cfu of *E. faecalis* JH2-2::I was inoculated to two groups of five germ-free mice supplied with water without or with vancomycin (Vm). Cocolonization in germ-free mice was performed by inoculating a 1:1 mixture of (*C*) *E. faecalis* JH2-2::IΩ*lucR* and JH2-2 or (*D*) JH2-2::C1Ω*lucR* and JH2-2::S. Results are expressed as the mean Log_{10} cfu/g of feces from two independent experiments.

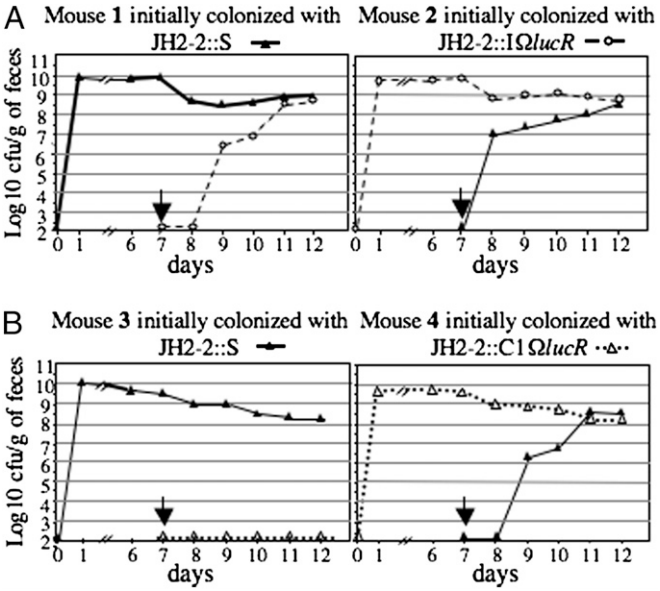

Fig. 4. Dissemination ability of *E. faecalis* strains in gnotobiotic mice. Sets of four mice were separately inoculated with 100 μL of *ca.* 10^8 cfu of *E. faecalis* JH2-2::S (mice 1 and 3), JH2-2::IΩ*lucR* (mouse 2), or JH2-2::C1Ω*lucR* (mouse 4). At day 7 (arrow), the mice colonized by JH2-2::S (mouse 1 or mouse 3) were housed in the same cage with either (*A*) a mouse colonized by JH2-2::IΩ*lucR* (mouse 2) or (*B*) a mouse colonized by JH2-2::C1Ω*lucR* (mouse 4). The fate of each strain was monitored by counting bacteria in daily collected fresh fecal samples from each mice. The results for each mice are expressed in Log_{10}cfu/g of feces.

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confirms that pairwise competition, which measures the change in the ratio of mixed strains during several successive growth phases (the lag, exponential, and stationary growth phases) is complementary to exponential growth rate comparison (1).

For in vitro growth rate comparison and competition experiments, susceptible *E. faecalis* JH2-2::S was generated by inactivation, after two point mutations, of the D,D-dipeptidase $vanX_B$ gene in JH2-2::I (Fig. 1) that is required for resistance (7). In the absence of vancomycin, JH2-2::I was as competitive as JH2-2::S, demonstrating that the biological cost of glycopeptide resistance in enterococci is negligible when noninduced. In contrast, a significant fitness reduction was observed in vitro when expression of the *vanB* operon was induced by vancomycin. In vivo, the intestinal colonization profiles of gnotobiotic mice by susceptible or inducible transconjugants were very similar (Fig. 3*A*). However, when the mice were treated with subinhibitory concentrations of vancomycin, there was a delay in colonization by the inducible strain, as well as a lower maintenance level (Fig. 3*B*). Thus, and as predicted, vancomycin resistance, when expressed, is biologically costly for enterococci. However, because the mechanism is very tightly regulated and expressed only in the presence of the inducer, the antibiotic itself, resistance is expressed exclusively when needed for bacterial survival (23). This strategy, in which the high fitness burden due to expression of resistance is largely compensated by the major competitive advantage provided to the host in the presence of the drug, accounts for worldwide dissemination of vancomycin resistance and its transfer to the major human MRSA pathogen (24). We have recently reported a similar significant reduction in growth rate (20–38%) of three VanA-type MRSA when expression of the *vanA* gene cluster was induced by vancomycin (25).

Constitutively resistant VanB-type enterococci are rarely found in clinical settings, and we have observed a marked fitness reduction in all such *E. faecalis* and *E. faecium* studied (Table 1). A major growth rate (Table 1) and competitiveness reduction (Table 2) was observed in constitutive strains in which, because of an impaired host Ddl ligase, only the resistance pathway is used for cell wall synthesis, indicating that the burden is related to the production of altered peptidoglycan. In the three constitutive derivatives of JH2-2::I, there is, in addition, production and removal by the cells of normal peptidoglycan precursors (Fig. S2). However, fitness reduction in these two different types of constitutive mutants was comparable and similar to the decrease observed in the inducible parental strain after induction by vancomycin (Table 1). This observation is in contrast with the finding that, in *E. coli*, the cost of expression of the *lac* operon proteins is associated with the process of making these proteins and not the products (26). The reason for the modified peptidoglycan biosynthetic pathway to impose a burden on the bacteria could be less efficient processing by the transpeptidases of late peptidoglycan precursors terminating in D-Ala-D-Lac compared with those ending in D-Ala-D-Ala, as has been proposed for PBP5 (27). Alternatively, the cost may result from a lower rate of production of pentadepsipeptide due to lower efficiency of some of the enzymes involved in D-Ala-D-Lac synthesis, addition of D-Ala-D-Lac to UDP-MurNAc-L-Ala-D-Glu-L-Lys, or of the amino acids involved in cross-linking to the precursor lipid intermediate during its transport across the membrane. The D,D-carboxypeptidase $VanY_B$ is important in the elimination of pentapeptide and may also hydrolyze, less efficiently, pentadepsipeptide. The peptidoglycan structure is unlikely to differ significantly between the susceptible and resistant strains because the terminal D-Ala or D-Lac are removed during cross-linking, although the degree of cross-linkage may be lower in the resistant strain.

Intestinal colonization by vancomycin-resistant enterococci is an important determinant of nosocomial infections with these pathogens, and the control of their spread requires a better understanding of their in vivo fitness. In cocolonization experiments, constitutive JH2-2::C1 was rapidly eliminated from the digestive tract of mice when administered with JH2-2::S (Fig. 3*D*) and could not disseminate to a mouse already colonized with this susceptible strain (Fig. 4*B*). This experimental study in animals showed that antibiotic resistance affects colonization and transmission rates. However, the most important findings were that inducible JH2-2::I was, in the absence of induction, not only able to cocolonize the intestine together with JH2-2 but also to disseminate to a mouse already colonized with the susceptible strain (Fig. 4*A*). These findings are consistent with the observation that vancomycin-resistant enterococci have been able to spread and persist in the absence of glycopeptides in the environment.

In contrast to VanB-type *E. faecalis*, there is a slight fitness burden due to the presence in VanA-type MRSA of noninduced transposon Tn*1546*. In these strains, and as opposed to the *vanB* operon in *Enterococcus* spp., expression of the *vanA* gene cluster is loosely regulated (6), and its carriage on multicopy plasmids results in a gene dosage effect that enhances the effect of loose regulation (25). These two findings result in a slight fitness disadvantage on the host that, in the absence of selective pressure, may account for the lack of dissemination of VanA-type vancomycin-resistant MRSA.

In conclusion, we have demonstrated in vitro and in vivo that neither Tn*1549* carriage nor noninduced resistance was significantly costly to the host, consistent with the worldwide dissemination of resistant enterococci. We have also shown that constitutive or induced resistance leads to a major reduction in growth rate, colonization, and transmission of *Enterococcus* spp. that could explain the low occurrence of constitutively resistant clinical isolates. These findings indicate that tight regulation of resistance mediated by a two-component regulatory system drastically reduces the biological cost associated with vancomycin resistance. Taken together, our findings account for the observation that expression of the majority of horizontally acquired antibiotic resistance mechanisms is tightly regulated (6) and that resistance determinants evolve to become selectively neutral in the absence of antibiotics.

Materials and Methods

Bacterial Strains, Plasmids, and Strain Construction. The origin and properties of the strains and plasmids are described in Table 1 and Table S1. Strains were grown in BHI.

Selection of constitutive mutants. *E. faecalis* JH2-2::I was grown overnight in broth and plated on agar containing teicoplanin (16 μg/mL). The mutants were screened for resistance to vancomycin and teicoplanin by disk diffusion, and the $vanR_B$ and $vanS_B$ genes of mutants JH2-2::C1, -::C2, and -::C3 were resequenced.

Construction of isogenic **E. faecalis** ***strains.*** In Gram-positive bacteria, pGhost9 (28), which replicates at 28 °C but is lost above 37 °C, allowed construction of *E. faecalis* JH2-2::I derivatives by insertional inactivation (Fig. S1). To obtain susceptible JH2-2::S, pAT889(pGhost9Ω*vanB′*$vanX_{B[A116H,\ A123D]}$360 bp) (Fig. S1 and Table S1 and Table S2) was electrotransformed into inducibly resistant JH2-2::I (Fig. 1). Plasmid pAT895(pGhost9Ω*int′*P_{tet}*lucRint″*) (Fig. S1 and Table S1 and Table S2) was electrotransformed into JH2-2::I, JH2-2::S, and JH2-2::C1 to generate, respectively, JH2-2::IΩ*lucR*, JH2-2::SΩ*lucR*, and JH2-2::C1Ω*lucR*. The integrants were screened for bioluminescence emission, and the integration locus was determined by PCR and sequencing.

Fitness Measurements. ***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 8 μg/mL of vancomycin. The cultures were diluted at OD 0.15 into 20 mL of broth without or with vancomycin (8 μg/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 two to four times in the same microplate. Growth rates performed in at least three independent experiments were determined at the beginning of the exponential phase, and relative growth rates were calculated as the ratio of the growth rate of the inducible or constitutive strains vs. susceptible strains.

Growth competition. Susceptible and resistant strains were grown separately at 37 °C in broth to an OD_{600} of 1, mixed in a 1:1 ratio, and diluted 1,000-fold (5×10^5 cfu) in 5 mL of broth. The mixed culture was transferred to fresh broth every *ca.* 10 generations over five cycles. The number of viable cells was determined at the end of every cycle by plating aliquots on nonselective

plates and on agar containing 8 μg/mL of vancomycin or 100 μg/mL of teicoplanin. The competition index (CI) was calculated as the cfu ratio of the resistant and susceptible strains at time (t_1) divided by the same ratio at time (t_0), and the selection coefficient *s* was then calculated as the slope of the following linear regression model: $s = \ln(CI)/[t \times \ln(2)]$, where t is the number of generations (29). Fitness of the susceptible strain was set to 1, and the relative fitness of the inducibly or constitutively resistant strains was determined as 1 + *s*.

Mice intestinal colonization. In vivo fitness was measured by colonization and cocolonization experiments in a gnotobiotic mice model. Six- to 8-wk-old C3H germ-free mice (Centre de selection des animaux de laboratoire, Orléans, France) were maintained in isolators, fed ad libitum with a commercial sterile diet, and supplied with autoclaved drinking water. For colonization, mice received intragastrically 100 μL of *ca.* 10^8 cfu in exponential growth phase after one wash in PBS. For cocolonization, susceptible and resistant bacteria were grown separately in broth, washed once in PBS, mixed in a 1:1 ratio, and *ca.* 10^5 cfu were inoculated intragastrically into C3H germ-free mice. Fresh fecal samples collected every day for 7 d were weighed, homogenized in PBS (0.1 mg/mL), diluted, and plated on agar without or with vancomycin (8 μg/mL). When the strain contained the *lucR* gene, the photons/s were measured, as described below, in various dilutions of the feces homogenized in PBS. The care and use of experimental animals complied with local animal welfare laws and guidelines.

Bioluminescence quantification. The thermostable PpyRE-TS firefly luciferase variant emits red light at 612 nm at 37 °C after exogenous addition of D-luciferin (30). Fifty microliters of serial dilutions of bacterial cultures or feces samples were inoculated in a 96-well black microplate, and 50 μL of D-luciferin (chromaGlo reagent; Promega) was added before measurement of bioluminescence. The microplates were placed in the warmed (37 °C) specimen chamber, and photon emission was measured using an IVIS 100 imaging system (Caliper Life Sciences) (17). The data appear as pseudocolor images indicating light intensity superimposed over the grayscale reference photographs. The region of interest was manually selected and the signal expressed as photons/s by using the IgorPro image analysis package.

Dissemination experiments. Mice were separately inoculated with 100 μL of *ca.* 10^8 cfu of various *E. faecalis* strains and were housed individually until the strains reached the maintenance stage of colonization. At day 7, the mice were then associated pairwise in the same cage. The fate of each strain was then monitored by collecting fresh fecal samples daily during 6 d. Dilutions of the feces were plated on agar, and the strains were differentiated by measuring bioluminescence emission from the colonies.

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