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Inducible and Constitutive Expression of *vanC-1*-Encoded Resistance to Vancomycin in *Enterococcus gallinarum*

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Clinical isolates *Enterococcus gallinarum* AIB39 and *E. gallinarum* GS1 were studied to establish whether the expression of *vanC-1*-mediated resistance may be inducible or constitutive. By growth curve analysis, strain AIB39 exhibited the same lag period (i.e., 1 to 1.5 h) whether it was subcultured to unsupplemented brain heart infusion broth or broth containing 6 μg of vancomycin per ml, a growth pattern typical of constitutively expressed resistance. Use of high-performance liquid chromatography (HPLC) to separate peptidoglycan precursor extracts substantiated this finding because the serine-terminating pentapeptide precursor UDP–MurNAc–L-Ala–D-Glu–L-Lys–D-Ala–D-Ser was produced in the presence and absence of vancomycin, whereas no UDP–MurNAc–L-Ala–D-Glu–L-Lys–D-Ala–D-Ala was detected. In contrast, results with strain GS1 were consistent with inducible expression. GS1 demonstrated a lag time that was 3 to 4 h longer when it was subcultured to vancomycin-containing broth than when it was subcultured in unsupplemented broth. HPLC analysis showed that in the absence of vancomycin only UDP–MurNAc–L-Ala–D-Glu–L-Lys–D-Ala–D-Ala was detected, but in the presence of drug only UDP–MurNAc–L-Ala–D-Glu–L-Lys–D-Ala–D-Ser was found. Inducible expression of *vanC-1*-mediated resistance in *E. gallinarum* is consistent with recent findings suggesting the presence of at least two ligases in this species. Although *vanC-1* may be intrinsic to *E. gallinarum*, our findings raise doubt regarding the natural mechanism of this gene's expression.

Acquired vancomycin resistance among enterococci, most usually associated with *Enterococcus faecium* and *Enterococcus faecalis*, is mediated by *vanA* and *vanB* genes that encode ligases capable of producing altered UDP-MurNAc-pentapeptide precursors for which vancomycin has diminished affinity (1). The vancomycin resistance exhibited by *Enterococcus gallinarum* is generally considered a constitutively expressed intrinsic characteristic of this species (1, 18, 27, 28). The intrinsic nature of this resistance is supported by the finding that the *vanC-1* gene, which encodes a ligase that catalyzes the formation of D-Ala–D-Ser, has consistently been found in every *E. gallinarum* strain examined and has not been found in other enterococcal species (9, 18, 22). However, recent findings suggest that although resistance may be intrinsic, expression may not always be constitutive (3, 22).

For other gram-positive organisms considered to be intrinsically and constitutively resistant, such as *Leuconostoc*, *Lactobacillus*, and *Pediococcus* species, evidence suggests the presence of a single ligase system (3, 15). The only peptidoglycan precursor found in these organisms is UDP–MurNAc–L-Ala–D-Glu–L-Lys–D-Ala–D-Lac (UDP–MurNac–tetrapeptide–D-Lac). In contrast, recent reports have established the likely existence of at least two ligases in *E. gallinarum*. This organism has exhibited the capacity to produce UDP–MurNAc–L-Ala–D-Glu–L-Lys–D-Ala–D-Ala (UDP–MurNac–tetrapeptide–D-Ala) cell wall precursor and the altered precursor MurNAc–L-Ala–D-Glu–L-Lys–D-Ala–D-Ser (UDP–MurNac–tetrapeptide–D-Ser), which is catalyzed by the *vanC-1*-encoded ligase (3, 22). The ability to produce the wild-type cell wall precursor UDP–MurNac–tetrapeptide–D-Ala and one with a decreased affinity for vancomycin (i.e., UDP–MurNac–tetrapeptide–D-Ser) is a feature that is strikingly similar to the inducible, acquired glycopeptide resistance encoded by *vanA* and *vanB*. In these systems induction results in the production of UDP–MurNac–tetrapeptide–D-Lac that has significantly reduced vancomycin affinity compared with that of the UDP–MurNac–tetrapeptide–D-Ala peptidoglycan precursor (1, 4, 10).

The presence of two ligases in *E. gallinarum*, a species heretofore considered to express resistance constitutively, is somewhat perplexing. In this report we establish the ability of *E. gallinarum* to inducibly or constitutively express *vanC-1*-mediated vancomycin resistance. Although *vanC-1* may be intrinsic to this species, these findings raise several questions regarding the intrinsic nature of the regulatory mechanisms that accompany *vanC-1*.

MATERIALS AND METHODS

Source, identification, and characterization of microorganisms. All enterococcal species were identified by the conventional scheme of Facklam and Collins (11), which included determination of motility at 30°C and the presence of yellow pigment. *E. gallinarum* GS1 is an isolate obtained in 1992 from the urine of a 73-year-old female with acute pyelonephritis seen at Good Samaritan Hospital, Downers Grove, Ill. *E. faecium* 228, *E. gallinarum* AIB39, *E. faecalis* V583, *E. faecalis* V583-2, *E. faecium* JB1, and *E. faecium* JB7 have been described previously (12, 15, 23, 24, 29). *E. faecalis* ATCC 29212 and *Enterococcus casseliflavus* ATCC 25788 were obtained from the American Type Culture Collection.

For each isolate, PCR was done to establish the presence of *vanA*, *vanB*, *vanC-1*, and *vanC-2*. The oligonucleotide primers selected for amplification, chosen from published sequences (7–10), were as follows: for *vanA* the sequence of the primer in the forward direction (bp 566) was 5′-GCTATTCAGCTGT ACTC-3′ and that of the primer in the reverse direction (bp 1332) was 5′-CAG CGGCCATCATACGG-3′, for *vanB* the sequence of the primer in the forward direction (bp 489) was 5′-CATCGCCGTCCCCGAATTTCAAA-3′ and that of the primer in the reverse direction (bp 765) was 5′-GATGCGGAAGATACCG TGGCT-3′, for *vanC-1* the sequence of the primer in the forward direction (bp 300) was 5′ GACCCGCTGAAATATGAAG-3′ and that of the primer in the reverse direction (bp 720) was 5′-CGGCTTGATAAAGATCGGG-3′, and for *vanC-2* the sequence of the primer in the forward direction (bp 455) was 5′-CT CCTACGATTCTCTTG-3′ and that of the primer in the reverse direction (bp 869) was 5′-GAATTTCCAGAACGAGC-3′.

Three to four bacterial colonies were taken from sheep blood agar plates

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following 18 to 24 h of incubation at 35°C and were suspended in 100 μl of the PCR mixture that contained 1× PCR buffer (500 mM KCl, 100 mM Tris-HCl [pH 9.0], 1% Triton X-100), 5 mM $MgCl_2$, 200 μM (each) deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dTTP), and 500 μM (each) primer. A Coy model 110s thermocycler (Coy Inc., Grass Lake, Mich.) was used to hot start the PCR mixture at 94°C for 5 min, after which 2.5 U of *Taq* DNA polymerase (Promega, Madison, Wis.) was added, with the thermocycler programmed as follows: 30 cycles of 1 min at 94°C, 1 min at 56°C, and 1 min at 72°C and then 2 min at 72°C; it was held at 4°C for analysis. For *vanC-1* and *vanC-2* PCR, 50°C rather than 56°C was used in the cycle. Amplification products were applied to 1.5% agarose gels for electrophoresis and were visualized by ethidium bromide staining.

Antimicrobial susceptibility testing and growth curves. Vancomycin and teicoplanin MICs were determined by the agar dilution method according to recommended guidelines (19). The susceptibility of each isolate to vancomycin was also tested by the agar screen method in which brain heart infusion (BHI) agar supplemented with 6 μg of vancomycin per ml is inoculated with 10^5 to 10^6 CFU (26).

As one method of establishing the constitutive or inducible expression of vancomycin resistance in *E. gallinarum*, growth curves were determined for strains GS1 and AIB39. Following overnight incubation in BHI broth, 1.5-ml aliquots from broth culture were harvested by centrifugation and were resuspended in 1.0 ml of fresh BHI broth. This suspension was used to inoculate 4.0 ml of BHI broth, unsupplemented and supplemented with 6 μg of vancomycin per ml, to a starting optical density at 620 nm of 0.05. The optical density at 620 nm of each culture was determined hourly for up to 8 h.

Peptidoglycan precursor analysis. Cytoplasmic pools of UDP-linked peptidoglycan precursors were extracted by a modification of a previously described method (14). Cultures were grown in BHI broth to the mid-logarithmic phase and were chilled rapidly, and the cells were harvested by centrifugation. Trichloroacetic acid (TCA) was added to a final concentration of 5%, and the mixture was incubated on ice for 30 min. After ether extraction to remove the TCA, the supernatant was adjusted to pH 7.0 with ammonium hydroxide and the contents of the supernatant were separated by gel filtration (Sephadex G-25; Pharmacia, Alameda, Calif.). Hexosamine-containing fractions were identified by the assay of Ghuysen et al. (12), pooled, and lyophilized. Precursors were separated by high-performance liquid chromatography (HPLC) by using a C_{18} μBondapak column (3.9 by 300 mm; Millipore-Waters, Milford, Mass.), with elution with 0.05 M ammonium formate (pH 4.65). Precursors were identified by comparison with the elution profile of the cytoplasmic pentapeptide precursor (UDP–MurNAc–L-Ala–D-Glu–L-Lys–D-Ala–D-Ala) obtained from *Staphylococcus aureus* 209P and by amino acid analysis as described previously (14).

Population studies, mating experiments, and selection of mutants. To investigate the homogeneous or heterogeneous nature of a strain's ability to grow when challenged with vancomycin, population analysis was done with *E. gallinarum* AIB39 and GS1. The previously characterized inducible VanA and VanB strains *E. faecium* 228, *E. faecalis* V583, *E. faecalis* V583-2, and *E. faecium* JB1 as well as the constitutive VanB isolate *E. faecium* JB7 were included for comparison. Overnight cultures grown at 35°C in BHI broth without vancomycin were serially diluted 10-fold so that colony counts of between 30 and 300 CFU could be accurately determined. Aliquots (0.1 ml) were taken from the dilution tubes and were plated onto BHI agar plates without vancomycin and onto plates supplemented with vancomycin at a concentration of one-fourth the MIC. Colony counts were done after 24 and 48 h of incubation. These population studies were done in triplicate for each strain.

Transfer of vancomycin resistance by *E. gallinarum* AIB39 and GS1 was studied by a previously described filter mating technique and with rifampin- and fusidic acid-resistant *E. faecalis* JH2-2 (5, 17). Additionally, the selection of mutants expressing higher-level resistance was attempted by growing strain AIB39 overnight in BHI broth and plating 0.1 ml of the broth culture (ca. 10^8 CFU) onto BHI agar plates containing 0.5 and 1.0 μg of teicoplanin per ml.

TABLE 1. Characteristics of enterococcal strains

Strain	MIC (μg/ml)		PCR product[a]	Reference or source
	Vancomycin	Teicoplanin		
E. gallinarum GS1	8	0.5	C-1	This study
E. gallinarum AIB39	8	0.5	C-1	23
E. faecium JB1	256	0.5	B	15
E. faecium JB7	>512	256	B	15
E. faecalis V583	64	0.5	B	23
E. faecalis V583-2	>512	0.5	B	29
E. faecium 228	>512	64	A	12
E. casseliflavus ATCC 25788	8	0.5	C-2	ATCC[b]

[a] A, *vanA*; B, *vanB*; C-1, *vanC-1*; C-2, *vanC-2*.
[b] ATCC, American Type Culture Collection.

RESULTS

Key characteristics of the strains included in the study are summarized in Table 1. Vancomycin and teicoplanin MICs were the same for both *E. gallinarum* AIB39 and GS1, and as expected for this species, each isolate yielded a PCR *vanC-1* product, while no *vanA*, *vanB*, or *vanC-2* product was detected. A *vanA* product was obtained with *E. faecium* 228. Similarly, a *vanB* product only was obtained with the VanB strains, which included *E. faecium* JB1, its constitutively resistant derivative JB7, *E. faecalis* V583, and its derivative V583-2; for strain V583-2 the vancomycin MIC is increased, but glycopeptide resistance is still inducible (15, 29). The susceptibility of *E. casseliflavus* ATCC 25788 to vancomycin and teicoplanin was the same as that expressed by the *E. gallinarum* strains, but the strain yielded only a *vanC-2* PCR product. Regardless of their genetic backgrounds, all isolates exhibited resistance, as determined by the agar screen method.

Comparison of resistance expression between *E. gallinarum* AIB39 and GS1 was first done by growth curve analysis. The results shown in Fig. 1 typify those obtained by multiple analyses generated for each isolate. AIB39 did not exhibit any notable difference in growth pattern whether it was subcultured to unsupplemented BHI broth or broth containing 6 μg of vancomycin per ml. In both cases a lag of ca. 1 to 1.5 h occurred, resulting in indistinguishable growth patterns typical of constitutive expression of resistance. In contrast, *E. gallinarum* GS1 exhibited a lag of 4 to 5 h when it was subcultured to

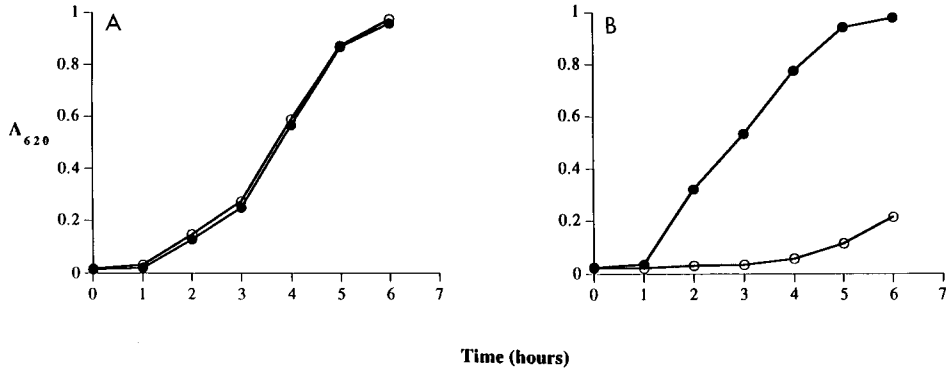

FIG. 1. Induction growth curves of *E. gallinarum* AIB39 (A) and *E. gallinarum* GS1 (B). ●, overnight growth in BHI broth and subculture in BHI broth; ○, overnight growth in BHI broth and subculture to BHI broth with 6 μg of vancomycin per ml.

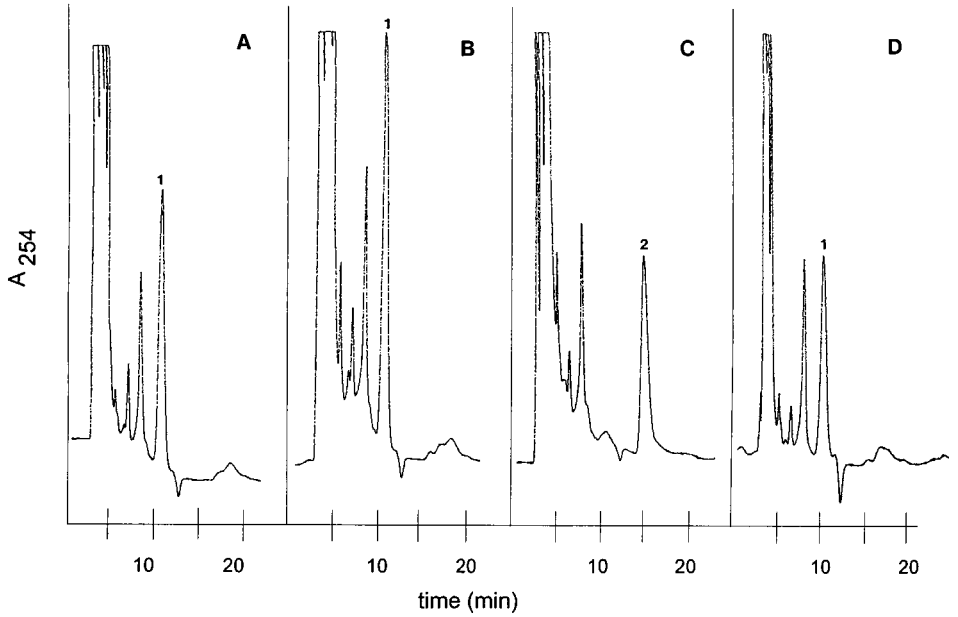

FIG. 2. HPLC separations of cytoplasmic pools of peptidoglycan precursors. Conditions used were absorbance units, full scale, 0.2; flow rate, 1 ml/min; elution with 0.05 M ammonium formate; pH 4.65. Each determination corresponds to approximately 20 nmol (as determined by the numbers of nanomoles of hexosamines). (A) *E. gallinarum* AIB39 grown without vancomycin; (B) *E. gallinarum* AIB39 grown with vancomycin (4 μg/ml); (C) *E. gallinarum* GS1 grown without vancomycin; (D) *E. gallinarum* GS1 grown with vancomycin (4 μg/ml). Peak 1, serine-terminating pentapeptide precursor; peak 2, D-Ala-terminating pentapeptide precursor.

BHI broth supplemented with vancomycin, resulting in a profile suggestive of inducible resistance.

Further evidence for differences in the control of resistance expression between *E. gallinarum* AIB39 and *E. gallinarum* GS1 was established by peptidoglycan precursor analysis (Fig. 2). HPLC separation of peptidoglycan precursor extracts from cultures of AIB39 demonstrated a peak that eluted earlier than the normal D-Ala-terminating pentapeptide precursor. On the basis of its elution profile and amino acid analysis, this peak was identified as the serine-terminating pentapeptide precursor (UDP–MurNac–L-Ala–D-Glu–L-Lys–D-Ala–D-Ser) described by Billot-Klein et al. (3). This peak was present in precursor extracts from cultures of AIB39 whether it was grown in the presence or the absence of 4 μg of vancomycin per ml (Fig. 2A and B). The normal D-Ala-terminating pentapeptide precursor was not detected under either growth condition (Fig. 2) or after cytoplasmic precursors had been accumulated by exposure of cultures to bacitracin (100 μg/ml) for 1 h prior to precursor extraction (data not shown). In contrast, only the normal D-Ala-terminating pentapeptide precursor was detected in extracts from cultures of strain GS1 grown in the absence of vancomycin (Fig. 2C). However, when it was grown in medium containing 4 μg of vancomycin per ml, the serine-terminating precursor was present and the normal D-Ala-terminating precursor was absent (Fig. 2D). No significant concentrations of UDP-MurNac-tripeptides were found in any of the precursor extracts.

Results of population studies by plating experiments are given in Table 2. For the previously characterized inducible VanA (*E. faecium* 228) and VanB (*E. faecalis* V583, *E. faecalis* V583-2, *E. faecium* JB1) strains the percentage of CFU on BHI agar containing vancomycin at one-fourth the MIC was less than 50%. Similarly, only 58% of *E. gallinarum* GS1 cells grew in the presence of vancomycin. The constitutively resistant *E. faecium* JB7 VanB strain did not exhibit a decrease in the number of CFU in the presence of vancomycin, and the decrease (6%) in the number of CFU for *E. gallinarum* AIB39 was negligible. To investigate the possibility that the numbers of CFU obtained with inducible strains might represent the selection of constitutive mutants, colonies were selected from vancomycin plates and were again subjected to the same protocol. In every instance the percentage of CFU on BHI agar containing vancomycin was comparable to that observed in the original test, indicating that growth was not due to the emergence of constitutive mutants. Population analysis of 26 other *E. gallinarum* isolates revealed a wide spectrum of results, with 13 strains exhibiting a ≥50% decrease in the numbers of CFU and 13 strains showing a <50% decrease in the numbers of CFU; one strain demonstrated a less than 10% decrease in the number of CFU (data not shown).

Transfer of vancomycin resistance to recipient *E. faecalis* JH2-2 could not be demonstrated with either *E. gallinarum* AIB39 or *E. gallinarum* GS1 (frequency, $<10^{-8}$ per donor cell). Selection of mutants expressing higher levels of glycopeptide resistance was also unsuccessful.

TABLE 2. Population analysis of vancomycin-resistant enterococci

Strain	Resistance expression[a]	CFU (10^7)[b]		% CFU[c]
		BHI agar	BHI agar with vancomycin	
E. faecalis V583	I	47	11	23
E. faecalis V583-2	I	122	41	34
E. faecium JB1	I	16	2	13
E. faecium 228	I	13	4	31
E. gallinarum GS1	I	50	29	58
E. faecium JB7	C	14	15	100
E. gallinarum AIB39	C	79	74	94

[a] I, inducible; C, constitutive.
[b] All CFU counts are averages obtained from three experiments.
[c] Percent CFU was calculated as (the average number of CFU on BHI agar with vancomycin/the average number of CFU on BHI agar) × 100.

DISCUSSION

Several lines of recent evidence indicate that two ligases are present in the purportedly constitutively vancomycin-resistant *E. gallinarum*. Dutka-Malen et al. (9) have shown that insertional inactivation of the *vanC-1* gene, which has subsequently been shown to encode a ligase that catalyzes synthesis of D-Ala–D-Ser (22), results in viable cells susceptible to glycopeptides. Additionally, peptidoglycan precursor analysis has demonstrated that some strains of *E. gallinarum* have the capacity to produce both the D-Ala- and the D-Ser-terminating precursors (3, 22). The need for two ligases in an organism that is constitutively resistant is not readily apparent. However, our finding that vancomycin resistance in *E. gallinarum* may be mediated by inducible production of the D-Ser-terminating precursor is consistent with the presence of at least two ligases.

Comparison of our precursor pool data with those of previous studies reveals a notable difference. In reports by Billot-Klein et al. (3) and Reynolds et al. (22), mixtures of D-Ala- and D-Ser-terminating precursors were evident when cells were grown in the presence or absence of vancomycin. In contrast, the constitutively resistant strain AIB39 exhibited no evidence of the D-Ala-terminating precursor, even in the absence of vancomycin. The absence of detectable pentapeptide precursor may be due to a lack of D-Ala–D-Ala ligase activity [i.e., a silent or absent gene(s)] or to efficient destruction of the D-Ala–D-Ala target, as has been described for the *vanA* system (2, 21). The lack of increased pools of UDP-MurNac-tripeptides in the AIB39 extracts suggests that DD-peptidase activity, which has been described in *E. gallinarum* BM4174 (22), may not have contributed significantly to the lack of a D-Ala-terminating precursor in AIB39. Unlike previously characterized strains of *E. gallinarum*, strain GS1 did not demonstrate any detectable production of the D-Ala-terminating precursor in the presence of vancomycin and did not demonstrate production of any D-Ser-terminating precursor in the absence of vancomycin. Whether the apparently complete switching between precursor pools reflects an efficient communication between the regulatory systems of the D-Ala–D-Ala ligase and the *vanC-1*-encoded D-Ala–D-Ser ligase (22) or efficient hydrolysis of the respective dipeptides remains to be determined.

Our findings raise several important issues concerning the evolution of vancomycin resistance in enterococci. Although *vanC-1* appears to be an intrinsic characteristic of *E. gallinarum* (18), the intrinsic nature of the regulatory system, or lack thereof, remains to be established. The regulatory system in strains such as *E. gallinarum* GS1 might be acquired from strains containing inducible *vanA* or *vanB* systems. This possibility is supported by a recent report of in vivo acquisition of *vanA* by *E. gallinarum* (6) and by the in vitro transferability of *vanA* from *E. faecium* to various enterococcal species, including *E. gallinarum* (23). The exact origin of this regulatory apparatus is still open to speculation.

Although there are similarities in inducible expression between *vanC-1* and *vanA* or *vanB*, some notable differences remain. The *vanC-1* gene is presumably intrinsic to *E. gallinarum*, while *vanA* and *vanB* are acquired (1, 3, 4, 10, 18, 22). Also, the constitutive strain AIB39 remains susceptible to teicoplanin, while constitutively resistant VanB strains are resistant to teicoplanin (16). Finally, selection of VanB, but not VanC, strains for which glycopeptide MICs are greater can be achieved by exposure to increasing concentrations of drug (16, 29, 30), and transfer of *vanC-1* resistance still has not been demonstrated.

The >50% decrease in the numbers of CFU of inducible *vanA* and *vanB* enterococcal populations obtained when the strains were challenged with subinhibitory concentrations of vancomycin has not been described previously. Our observation that the constitutive VanB mutant JB7 did not show a decrease in the numbers of CFU while the parent strain JB1 did suggest that this difference is related to inducibility. The comparable decrease in the numbers of CFU exhibited by *E. gallinarum* GS1, but not by *E. gallinarum* AIB39, is consistent with the inducible and constitutive nature of these strains, respectively. With the marked decrease in the numbers of CFU used as a screen for inducible resistance, it is interesting that approximately half of the 26 other *E. gallinarum* strains studied had a ≥50% decrease in their numbers of CFU when they were challenged with vancomycin. These observations suggest that inducible *vanC-1*-mediated resistance is not rare. Also of note, similar results were obtained with 10 of 11 *E. casseliflavus* strains studied, and growth curve analysis strongly suggests that vancomycin resistance in this species frequently may be inducibly expressed (data not shown). These findings are consistent with the recent report by Navarro and Courvalin (20) describing the presence of two ligase genes, $ddl_{\text{E.cass}}$ and *vanC-2*, encoding Ddl-related enzymes in this enterococcal species.

From a clinical perspective, the potential impact of inducible versus constitutive expression of *vanC-1* on the therapeutic effectiveness of vancomycin is not clear. No effect on the level of resistance was noted, since vancomycin MICs for AIB39 and GS1 were identical. Furthermore, the impact on in vitro susceptibility testing by the vancomycin agar screen is likely negligible, because all *E. gallinarum* strains studied were able to grow in the presence 6 μg of vancomycin per ml (data not shown). Nonetheless, establishing the existence of both constitutive and inducible *vanC-1*-mediated resistance provides important information for further studies focused on the eventual elucidation of the emergence, evolution, and dissemination of glycopeptide resistance.

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