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Cloning of the *Staphylococcus aureus ddh* Gene Encoding NAD$^+$-Dependent D-Lactate Dehydrogenase and Insertional Inactivation in a Glycopeptide-Resistant Isolate

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The mechanism of low-level glycopeptide resistance among staphylococci is not known. A cytoplasmic protein, provisionally called Ddh (W. M. Milewski, S. Boyle-Vavra, B. Moreira, C. C. Ebert, and R. S. Daum, Antimicrob. Agents Chemother. 40:166–172, 1996), and the RNA transcript that contains the *ddh* gene, which encodes Ddh, are present in increased amounts in a vancomycin-resistant isolate, 523k, compared with the susceptible parent isolate, 523. Sequence analysis had previously revealed that Ddh is related to NAD$^+$-dependent D-lactate dehydrogenase (D-nLDH) and VanH. This latter protein is essential for high-level glycopeptide resistance in *Enterococcus faecium* and *Enterococcus faecalis* by synthesizing the D-lactate needed for biosynthesis of D-lactate-terminating peptidoglycan precursors with low affinity for vancomycin. We now provide the direct evidence that the *ddh* gene product is *Staphylococcus aureus* D-nLDH and hereafter refer to the protein as D-nLDH. However, overproduction of this protein in isolate 523k did not result in production of D-lactate-containing peptidoglycan precursors, and susceptibility testing of *ddh* mutants of 523k demonstrated that *S. aureus* D-nLDH is not necessary for glycopeptide resistance in this isolate. We conclude that the mechanism of glycopeptide resistance in this isolate is distinct from that in enterococci.

The glycopeptide antimicrobials vancomycin and teicoplanin inhibit the transglycosylation and transpeptidation reactions that are required for synthesis of peptidoglycan by binding to the D-alanyl-D-alanine (D-Ala-D-Ala) termini of peptidoglycan pentapeptide precursors (reviewed in reference 6). Despite the identification of several properties that differ between susceptible staphylococcal isolates and those with decreased susceptibility or resistance to glycopeptides (8, 13, 27, 31, 35, 40), the mechanism mediating glycopeptide resistance in any staphylococcal isolate remains unknown.

To investigate the vancomycin resistance mechanism in *Staphylococcus aureus*, we (13, 31) and others (7, 20, 27, 44) have produced isolates with decreased susceptibility (resistance) to vancomycin and teicoplanin by serial incubation of susceptible clinical isolates in the presence of increasing concentrations of vancomycin. In one such glycopeptide-resistant isolate, 523k, derived from the susceptible clinical isolate 523, the MICs of both vancomycin and teicoplanin increased above the susceptibility breakpoints and no change occurred in the susceptibility of any other antimicrobial tested (13). Moreover, the resistance phenotype was stable in the absence of glycopeptides (13).

By nucleotide and peptide sequencing, we recently identified a 37-kDa cytoplasmic protein, provisionally called Ddh, that is constitutively overproduced by isolate 523k (13, 30). Ddh is related (30) to the family of bacterial D-2-hydroxyacid dehydrogenases, which includes NAD$^+$-linked D-lactate dehydrogenases (D-nLDH by the nomenclature of Garvie [18]) and the VanH dehydrogenase (2), which is essential for acquired high-level vancomycin resistance in *Enterococcus faecium* and *Enterococcus faecalis* (1). The role of VanH in vancomycin resistance in enterococci which harbor the *vanH* gene is to synthesize D-lactate, which becomes the carboxyl-terminal residue of the stem peptide portion of peptidoglycan precursors (11). The presence of D-lactate instead of the usual D-Ala residue at the terminal position decreases the affinity of the precursor for vancomycin 1,000-fold (11).

There are a few bacterial species that have low-level intrinsic glycopeptide resistance by producing a small proportion of peptidoglycan precursors terminating in D-lactate or serine, in addition to the wild-type precursor, which terminates in D-Ala (9, 22). In some of these bacteria, such as *Lactobacillus casei*, the lactate produced for metabolic purposes is presumably funneled into the production of the D-lactate-terminating precursors. Similarly, in a vancomycin-resistant *Staphylococcus haemolyticus* isolate, although the bulk of the total pool of peptidoglycan precursors terminate in D-Ala, a small fraction terminate in D-lactate (8).

The observation that increased cytoplasmic D-nLDH activity corresponded to increased production of the 37-kDa protein in *S. aureus* isolate 523k (30) strongly suggested that this protein is the *S. aureus* D-nLDH described previously (19) and provided a possible explanation for the low-level glycopeptide resistance in the isolate. We speculated that in *S. aureus* isolates with high amounts of D-nLDH activity, particularly isolate 523k, glycopeptide resistance could be due to a portion of the isolate's peptidoglycan precursors terminating in D-lactate. To gain further evidence for this hypothesis, we cloned and expressed the *ddh* gene in *Escherichia coli* and insertionally inactivated it in the chromosome of strain 523k. These studies confirmed that the *ddh* gene product is the cytoplasmic D-

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TABLE 1. Plasmids used in this study

Plasmid	Description	Source or reference
pBluescript II SK$^+$	*E. coli* cloning vector	Stratagene
pPQ126	Source vector for the pTV1$_{ts}$ Ts *ori* and the *aac*/*aph* gentamicin resistance cassette	28
pM952	pBluescriptII SK$^+$ vector containing a 952-bp PCR product containing the 5′ portion of the *ddh* gene from 523k	30
pM952-1	pM952 containing an *aac*/*aph* insertion in the *ddh* gene	This study
pM952-2	pM952-1 containing a Ts *ori* from pTV1$_{ts}$	This study
pddh	pBluescriptII SK$^+$ containing a 1.4-kb insert with the entire *ddh* gene from 523k	This study (Fig. 1)

nLDH enzyme in *S. aureus*, and therefore Ddh is hereafter referred to as D-nLDH. However, overproduction of D-nLDH in 523k did not result in production of D-lactate-terminating peptidoglycan precursors and was not necessary for glycopeptide resistance. To resolve some ambiguity in the literature concerning the role of this protein in glycopeptide resistance in staphylococci, we also analyzed D-nLDH activity in a teicoplanin-resistant *S. aureus* clinical isolate which produces a novel 35-kDa membrane protein (40) and in a vancomycin-resistant *S. haemolyticus* isolate which produces a small amount of D-lactate-terminating peptidoglycan precursors (23).

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MATERIALS AND METHODS

Plasmids, strains, and susceptibility testing. All plasmids used in this study are listed in Table 1. Growth and storage of strains was performed as previously described (30). When indicated, ampicillin (Sigma, St. Louis, Mo.) (100 μg/ml), chloramphenicol (10 μg/ml), gentamicin (10 μg/ml), kanamycin (40 μg/ml), or 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) (Gibco BRL) (40 μg/ml) was added to the medium. Determinations of the vancomycin MIC were performed by macrodilution of cultures in 24-well plates (Costar) with an inoculum size of 10^5 CFU/ml in Mueller-Hinton broth (Difco, Detroit, Mich.) adjusted with Ca^{2+} and Mg^{2+} cations according to the recommendations of the National Committee for Clinical Laboratory Standards (33). To detect subtle differences in MICs among the strains, single incremental increases in the concentrations of vancomycin and teicoplanin were tested (ranging from 1 to 5 μg/ml). Time-kill kinetic studies were performed at a vancomycin concentration of 4 μg/ml as previously described (13).

Vancomycin-resistant *S. aureus* derivatives were obtained as previously described (13) by successive broth incubation of susceptible clinical isolates in increasing concentrations of vancomycin. The mutant *S. aureus* isolate 523k with decreased susceptibility to vancomycin (MIC, 5 μg/ml) and teicoplanin (MIC, 5 μg/ml) was isolated previously from clinical isolate 523 (MICs of vancomycin and teicoplanin, 0.6 and 1 μg/ml, respectively) (13). The *S. aureus* clinical isolates 12871 and 12873 (25, 40) were provided by Glenn Kaatz (Detroit Receiving Hospital, Detroit, Mich.). *S. haemolyticus* isolate 18-88 (vancomycin MIC, 16; teicoplanin MIC, 32) was derived (23) by laboratory passage of susceptible clinical isolate 18 (vancomycin MIC, 4; teicoplanin MIC, 8) on vancomycin and was provided by Loreen Herwaldt (University of Iowa, Iowa City). *S. aureus* glycopeptide-susceptible isolates 1714 and 1715 are β-lactamase-negative clinical blood isolates obtained from the Clinical Microbiology Laboratory at the University of Chicago. *S. aureus* isolate 1729 is ATCC reference strain 25923. *S. aureus* RN4220, used as the nuclease-negative intermediate host strain for cloning of genes between *E. coli* and *S. aureus* (26), was provided by Jean Lee (Harvard Medical School, Boston, Mass.).

E. coli DH5α was used as the host for all *E. coli* cloning procedures. *E. coli* NZN111, provided by David P. Clark (Southern Illinois University, Carbondale, Ill.), was constructed from a derivative of LCB320 by insertion of a kanamycin resistance cassette within the *ldhA* gene (which encodes cytoplasmic D-nLDH; GenBank accession no. U36928) and of a chloramphenicol resistance cassette within the *pfl* gene (which encodes pyruvate formate lyase), and therefore it lacks both cytoplasmic D-nLDH and pyruvate formate lyase activities (12a, 15).

Northern and Southern blotting. To prepare RNA for Northern blotting, overnight cultures were harvested by centrifugation at 4°C. To disrupt cell walls, the cell pellets were gently resuspended in 500 μl of Tris-EDTA (TE) buffer (pH 8.0) containing lysostaphin and were incubated 5 to 10 min at room temperature. To adjust for differences in lysostaphin susceptibility of the strains (13), lysostaphin was used at concentrations of 100 μg/ml for isolate 523 and 500 μg/ml for isolate 523k. Whole-cell RNA was isolated by using the RNeasy kit (Qiagen) according to the manufacturer's instructions. The resulting RNA was ethanol precipitated (39) and resuspended in diethyl pyrocarbonate-treated water, and RNasin (Boehringer Mannheim) was added before storage at −70°C. Electrophoretic separation of RNA (5 μg) and Northern blotting techniques were performed as described previously (39), with hybridization performed in the presence of formamide at 42°C. The RNA concentration was determined from the optical density at 260 nm, and the purity of the RNA was established from the A_{260}/A_{280} ratio. To ensure that equivalent amounts of RNA from 523 and 523k were compared, the intensities of the rRNA bands were visualized in the gel by ethidium bromide staining. Southern blotting of chromosomal DNA was performed as described previously (39), with hybridization performed at 65°C.

Radiolabeled hybridization probes were prepared as described previously (16) by electrophoretic separation of restriction fragments in low-melting-point agarose and random priming with [α-^{32}P]dCTP or [α-^{32}P]dATP (Amersham) and the RadPrime DNA labeling kit (Gibco BRL). Unincorporated nucleotides were removed from the probe by centrifugation over a CL-6B spin column as described in "DNA manipulations, cloning, and DNA transformation" below. The *ddh* probe was the 0.9-kb *Eco*RI-*Hin*dIII fragment of pM952 containing the partial *ddh* gene (30). To detect the gentamicin resistance cassette, the 600-bp *Hpa*I-*Sca*I restriction fragment from the *aacA*/*aphD* gene of Tn*4001* isolated from pPQ126 was used (28).

DNA manipulations, cloning, and DNA transformation. Chromosomal DNA from *S. aureus* was isolated as described previously (32). Plasmid DNA was isolated with the use of Wizard Plus plasmid midiprep or miniprep kits (Promega, Madison, Wis.). For isolation of plasmid DNA from *S. aureus*, the protocol was modified by adding lysostaphin (50 to 1,000 μg/ml) to the resuspension buffer and performing lysis for at least 30 min at 37°C. Restriction enzyme digestions, blunting of DNA ends with T4 DNA polymerase, and ligations with T4 DNA ligase were all performed according to standard procedures (5). To accept PCR products, a thymine tail was added to blunt-ended pBluescript II SK$^+$ (Stratagene) as described previously (5). All enzyme reaction mixtures used in cloning procedures were purified by centrifugation through Sepharose CL-6B spin columns produced in microcentrifuge tubes as follows: a 60% slurry of TE-equilibrated CL-6B (pH 8.0) (obtained as an ethanol suspension from Sigma) was applied to a small silanized glass-bead layer and centrifuged in an amount equal to 15 times the sample volume at 2,500 rpm for 2 min in a Beckman centrifuge (model TJ-6); samples were passed through the columns at the same speed for 2 min.

Electrocompetent *E. coli* cells were prepared and electroporated as described previously (5) in a 0.1-cm electroporation cuvette, except that an Invitrogen electroporator (model II) (San Diego, Calif.) was used at settings of 1,500 V, 50 μF, and 70 Ω. Electrocompetent *S. aureus* cells were prepared and electroporated as described previously (4), except that the Invitrogen electroporator (model II) was used at settings of 1,800 V, 50 μF, and 70 Ω with a 0.2-cm cuvette.

Cloning of the *ddh* gene. The strategy used to construct pddh, containing the 523k *ddh* gene, is depicted in Fig. 1. The 1,431-bp PCR product containing the full-length *ddh* gene was amplified from strain 523k as described previously (30), by use of a primer pair (primers 3 and 4) previously designed (30) to hybridize to noncoding sequences flanking the 5′ and 3′ termini of the *ddh* open reading frame (ORF). The PCR was used in a ligation reaction with *Eco*RV-digested pBluescript II SK$^+$ (Stratagene) modified by the addition of a thymine residue to the 3′ termini as described elsewhere (5). Initial attempts at transforming *E. coli* with the ligation yielded plasmids with inserts of an unexpected size; the pddh clone containing the full-length PCR product was obtained when the incubation time for expression of ampicillin resistance was decreased from 1 h to 15 min and transformants were plated onto medium containing 20 mM lithium D(−)-lactate (Boehringer Mannheim).

Western immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, preparation of the anti-Ddh (hereafter referred to as anti-D-nLDH) polyclonal antiserum, and conditions for its use in immunoblotting have been described previously (13, 30). The primary antibody was detected with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Sigma) treated with BCIP (5-bromo-4-chloro-3-indolyl phosphate) (Sigma) and nitroblue tetrazolium (NBT) (Sigma). Molecular masses of proteins detected on the Western blots were estimated by comparison with S · tagged protein standards (Novagen) which were visualized directly on the nitrocellulose as described by the manufacturer.

D(−)-nLDH assays. To prepare *S. aureus* extracts, cells from overnight cultures were lysed by either lysostaphin treatment (final concentration, 100 μg/ml in 50 mM KH_2PO4, [pH 7.4]) or sonication (1-s pulses at 50% output performed for 15 min). The cytoplasmic and membrane fractions were obtained by differential centrifugation as described previously (30). D-nLDH activity was determined by measuring the rate of production of NADH in the presence of D-lactate (30). Specific activity is reported as micromoles of NADH produced per minute

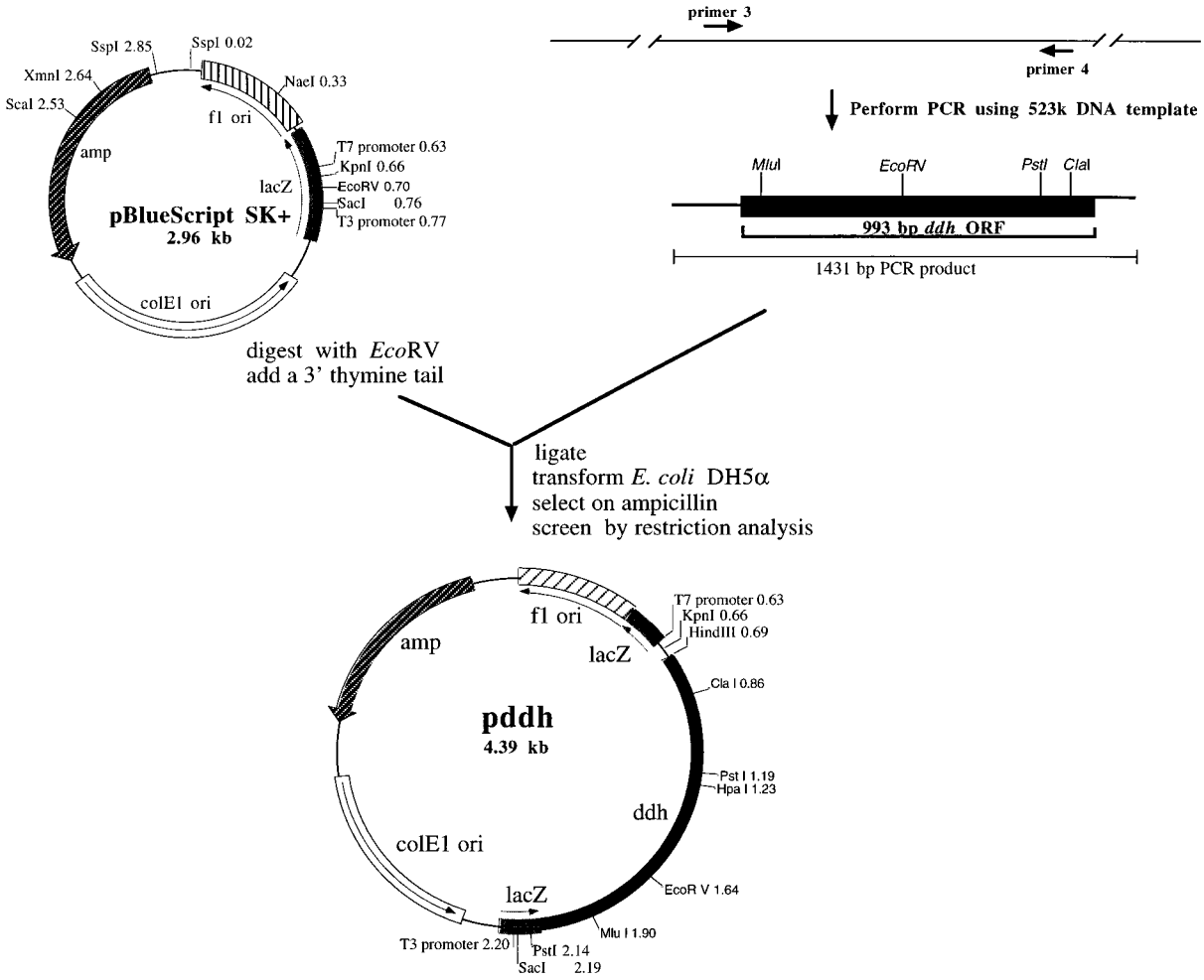

FIG. 1. Construction of pddh. The 1,431-bp PCR product containing the entire *ddh* gene obtained from isolate 523k was inserted into the *Eco*RV site of pBluescriptII SK$^+$.

per milligram of protein. Assays were performed in triplicate on at least two separate lysate preparations.

Insertional inactivation of *ddh* in strain 523k. The allelic replacement vector and the strategy for insertional inactivation of *ddh* are shown in Fig. 2. A 952-bp fragment containing the partial *ddh* gene from isolate 523k was previously cloned in pBluescriptII SK$^+$ and was designated pM952 (30). The 2.5-kb fragment containing the Tn*4001*-derived *aacA*/*aphD* gene encoding gentamicin resistance was obtained from the vector pPQ126 (28) by digestion with *Hin*dIII; digestion products were then blunt ended and ligated with pM952 which had been linearized at the *Eco*RV site internal to the *ddh* gene (positioned 281 nucleotides from the start of the ORF) (30). Transformants were obtained in *E. coli*, and plasmid pM952-1 containing the *aac*/*aph* gene was identified by restriction analysis. The temperature-sensitive origin of replication (Ts *ori*) derived from the *S. aureus* plasmid pTV1$_{ts}$ (43) was excised from pPQ126 (28) (provided by Joseph Patti, Texas A & M University, Houston, Tex.) by *Hpa*I digestion and was ligated with *Not*I-digested and blunt-ended pM952-1. pM952-2 was isolated after electrotransformation of *S. aureus* RN4220, selection on gentamicin, and confirmation of plasmids which contained the Ts *ori* by restriction analysis.

In order to perform allelic exchange, pM952-2 was isolated from RN4220 by use of CsCl-ethidium bromide density gradients (34) and 1 μg of DNA was electroporated into strain 523k. In order to select recombinants which contained an integrated copy of the gentamicin resistance gene in the chromosomal *ddh* locus, the pool of transformants was passaged as described previously (37) in the presence of gentamicin at the nonpermissive temperature (42°C) for Ts *ori*-directed replication.

Peptidoglycan precursor isolation and mass spectrometry. UDP-linked *N*-acetyl muramyl peptidoglycan precursors were prepared as described previously (21) from mid-exponential-phase broth cultures of strains 523 and 523k. To enrich for precursors, 523k was grown in the presence of 1/4 the vancomycin MIC. Briefly, a crude separation of the precursors was performed by size exclusion chromatography over Sephadex G-25. The precursor-containing fractions were pooled, subjected to reverse-phase high-performance liquid chromatography (RP-HPLC), and detected at an optical density of 254 nm (21).

RESULTS

Increased abundance of the *ddh*-specific transcript in isolate 523k. To examine the basis for increased production of D-nLDH in isolate 523k, Northern blot analysis was performed with the *ddh* probe and RNA obtained from stationary-phase cultures of 523k and the susceptible parent isolate, 523 (Fig. 3). The *ddh* probe detected a 1.2-kb transcript in both isolates. The size of the transcript is similar to that of the gene (30), a finding suggesting that the *ddh* gene is monocistronic. Consistent with the relative production of the D-nLDH protein in these isolates (30), the abundance of the *ddh* transcript in 523k far exceeded that of isolate 523. We conclude, therefore, that an increase in the abundance of the *ddh* message can explain the increased production of this protein in the resistant isolate.

Cloning and characterization of the full-length *ddh* gene. The sequence of the *ddh* gene was previously obtained from two clones containing separate but overlapping PCR products, each carrying a portion of the gene from strain 523k (30). To confirm that the *ddh* gene encodes the overproduced cytoplasmic protein in strain 523k and that the overproduction was

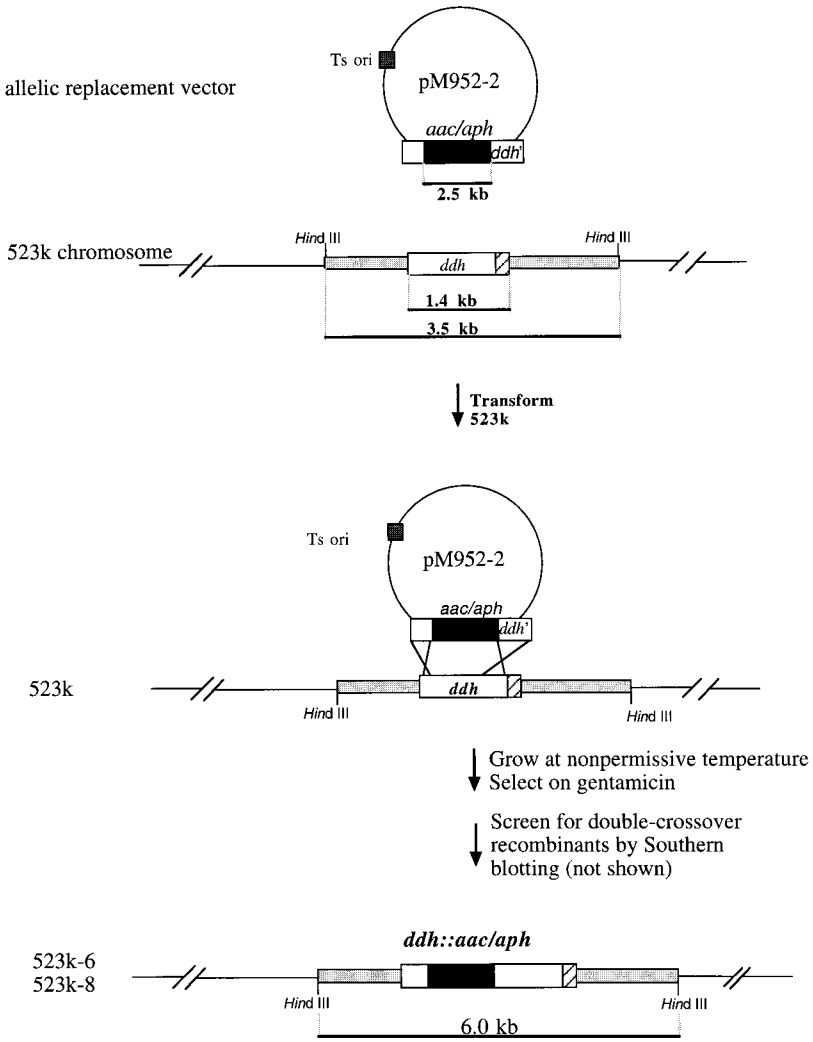

FIG. 2. Allelic replacement strategy for inactivation of the *ddh* gene in isolate 523k. The allelic replacement vector (pM952-2) contains the $pTV1_{ts}$ Ts *ori* and a 952-bp fragment consisting of a portion of the *ddh* gene (open box) interrupted by a 2.5-kb gentamicin resistance cassette (*aac*/*aph*) (solid box). pM952-2 was targeted to recombine with the 1.4-kb *ddh* locus contained on a 3.5-kb *Hin*dIII fragment in the chromosome of 523k (*ddh* sequences contained in the chromosome that are not present in pM952-2 are shown as a box with diagonal lines). Growth in the presence of gentamicin at the nonpermissive temperature selected for recombinants expressing gentamicin resistance from the *ddh* locus in the chromosome. The inactivation mutants 523k-6 and 523k-8 identified by Southern blotting have a new 6-kb *ddh*-specific fragment containing the gentamicin resistance cassette.

responsible for increased D-nLDH activity (30), the full-length *ddh* gene was cloned (Fig. 1) and expressed in *E. coli*.

Initially, when a λgt11 library of *S. aureus* FDA485 was screened by either plaque hybridization with a *ddh* specific probe or plaque immunoblotting with the anti-D-nLDH antiserum, clones containing only a portion of the *ddh* gene were obtained. Therefore, the full-length *ddh* gene was isolated and cloned by PCR amplification of a 1,431-bp fragment from strain 523k with a primer pair designed to anneal with 5′ and 3′ noncoding regions that flank the putative promoter and transcriptional stop site, respectively, of the *ddh* gene (30). An *E. coli* transformant, designated T5-17, was identified that harbored a plasmid, called pddh (Fig. 1), consisting of pBluescript containing an insert of the expected size. Western blotting with the anti-D-nLDH antiserum as a probe revealed that T5-17, but not DH5α harboring pBluescript, produced a band comigrating with the D-nLDH protein from *S. aureus* 523k (data not shown).

To determine whether the *ddh* gene product could express D-nLDH activity, pddh was transformed into *E. coli* NZN111, which contains a kanamycin resistance cassette disrupting the *ldhA* gene, which encodes the cytoplasmic D-nLDH of *E. coli* (12, 41, 42). As expected, a 29-fold mean increase in D-nLDH activity was present in NZN111(pddh) compared with the par-

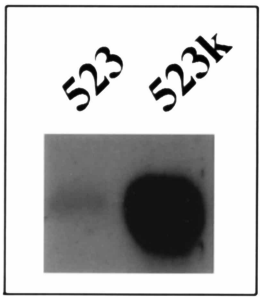

FIG. 3. Northern blot of RNA obtained from isolates 523 and 523k probed with the radiolabeled *ddh* gene probe.

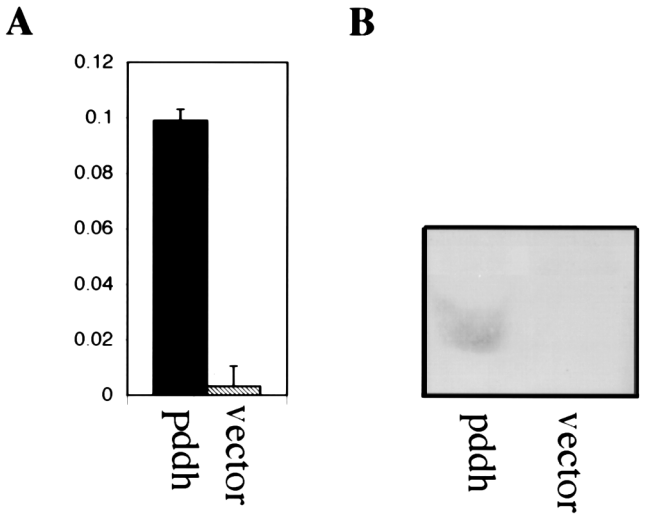

FIG. 4. Expression of the *ddh* gene in *E. coli*. (A) D-nLDH specific activity (micromoles of NADH produced per minute per milligram of protein) in NZN111 harboring pddh or vector. Two experiments were performed in triplicate. Error bars, standard deviations. (B) Western blot of cytoplasmic fractions from NZN111 harboring pddh or vector probed with the anti-D-nLDH antiserum. The band in the pddh lane comigrates with the band from 523k (data not shown).

ent strain containing the vector (Fig. 4A). Also, the presence of pddh in NZN111 resulted in the production of a protein band (Fig. 4B) which comigrated with D-nLDH from 523k (data not shown) in Western blots probed with the anti-D-nLDH antiserum. These data demonstrate that D-nLDH is encoded by the *ddh* gene.

Inactivation of the *ddh* gene in 523k. To investigate the possibility that production of D-nLDH is necessary for the glycopeptide resistance phenotype, the *ddh* gene in the chromosome of the vancomycin-resistant strain 523k was inactivated by allelic replacement. The allelic replacement vector, pM952-2 (Fig. 2), was constructed by inserting the *aacA/aphD* gentamicin resistance gene in the partial *ddh* ORF contained in plasmid pM952 and adding the $pTV1_{ts}$ Ts *ori* to permit suicide delivery of the vector into *S. aureus* (Fig. 2). For insertional inactivation, a transformant of 523k containing pM952-2, designated 523k-14, was grown at the permissive temperature to permit homologous pairing and recombination between the chromosomal *ddh* locus and the plasmid-encoded *ddh* sequences flanking either side of the gentamicin resistance cassette. Recombinants which expressed gentamicin resistance from a chromosomal location were selected by growing cultures at the nonpermissive temperature for Ts *ori*-directed plasmid replication in the presence of gentamicin.

Southern blotting was used to screen the recombinants for those that contained a *ddh* gene that was disrupted by the gentamicin resistance cassette in the chromosome (data not shown). In two isolates, designated 523k14-6 and 523k14-8, the single 3.5-kb *ddh*-specific *Hin*dIII fragment present in isolates 523 and 523k was increased by 2.5 kb, the size of the gentamicin resistance cassette. This new 6.0-kb fragment cross-hybridized with the *aacA/aphD* gene probe and therefore contained the gentamicin resistance cassette. No other fragments hybridized with the *ddh* or *aacA/aphD* probes. These data indicate that these recombinants resulted from a double-crossover homologous recombination event between plasmid and chromosomal *ddh* sequences resulting in the insertion of the gentamicin resistance cassette into the chromosomal *ddh* locus (Fig. 2).

Confirming that *ddh* was inactivated in 523k14-6 and 523k14-8 was the fact that no increase in NADH production was seen following addition of D-lactate to cytoplasmic extracts of these isolates, in contrast to the parent 523k, where a clear increase occurred (Fig. 5A). Also, Western blotting confirmed that D-nLDH was not detectable in 523k14-6 and 523k14-8 (Fig. 5B). Thus, D-nLDH activity and production in both *ddh* inactivation mutants were undetectable, as was the case with susceptible isolate 523.

As shown in Table 2, the MICs of vancomycin and teicoplanin for 523k(pM952-2) and the two *ddh* inactivation mutants were identical. Likewise, the time-kill kinetics in the presence of 4 μg of vancomycin/ml were identical for strains 523k and 523k14-6 (data not shown). These analyses indicate that D-nLDH overproduction is not essential for glycopeptide resistance in 523k.

Effect of D-nLDH overproduction on peptidoglycan precursor composition in 523k. To investigate whether increased D-nLDH activity in *S. aureus* 523k could lead to production of peptidoglycan precursors containing a carboxyl-terminal D-lactate, UDP-linked cytoplasmic peptidoglycan precursors from isolates 523 and 523k were analyzed by RP-HPLC (Fig. 6) (21). No peak was detected at the retention time of the D-lactate-terminating peptidoglycan precursor standard in the chromatogram for either strain (Fig. 6). A peak which eluted with a retention time identical to that of the D-Ala-terminating peptidoglycan precursor standard (Fig. 6) was present in both 523 and 523k. As determined by mass spectroscopy analysis, the molecular mass of the material eluting in this peak in both strains was 1,149 Da, a value which was the same as that determined for the D-Ala-terminating standard (data not shown). Furthermore, amino acid analysis of the suspected D-Ala-terminating precursor fraction confirmed the usual structure (UDP-MurNAc-Ala-Glu-Lys-Ala-Ala) for peptidoglycan precursors in both 523 and 523k. Whereas the peaks containing the UDP-linked D-Ala-D-Ala containing precursors disappeared from the chromatogram after boiling in 0.05 M HCl, the other peaks which were dissimilar between isolates were still detected and therefore do not represent nucleotide-linked cytoplasmic peptidoglycan precursors. These data indicate that the peptidoglycan precursor pool in 523k does not contain a species which terminates in D-lactate.

Assessment of D-nLDH production and activity in staphylococcal isolates with decreased susceptibility to glycopeptides. To assess the relationship between D-nLDH activity and the glycopeptide resistance phenotype in staphylococci, cytoplasmic D-nLDH activity was measured in glycopeptide-resistant isolates and in susceptible parent strains. Isolates examined were (i) a teicoplanin-resistant clinical *S. aureus* isolate, 12873, that produces a 35-kDa membrane protein not apparent in the related susceptible isolate 12871 (40); (ii) a laboratory-derived vancomycin-resistant *S. haemolyticus* isolate, 18-88, which produces a small amount of D-lactate-terminating peptidoglycan precursors along with the large pool that terminate in D-Ala, and its susceptible parent isolate, 18 (8); and (iii) nine laboratory-derived vancomycin-resistant *S. aureus* isolates and the respective related susceptible isolates 523, 1715, 1714, and 1729.

The vancomycin-susceptible–vancomycin-resistant isolate pairs of *S. aureus* 12871 and 12873 and *S. haemolyticus* 18 and 18-88 each produced high levels of D-nLDH activity, comparable to that of 523k. Most susceptible isolates produced measurable activity, and three susceptible isolates (1714, 12871, and 18) had higher activity than at least one of their respective

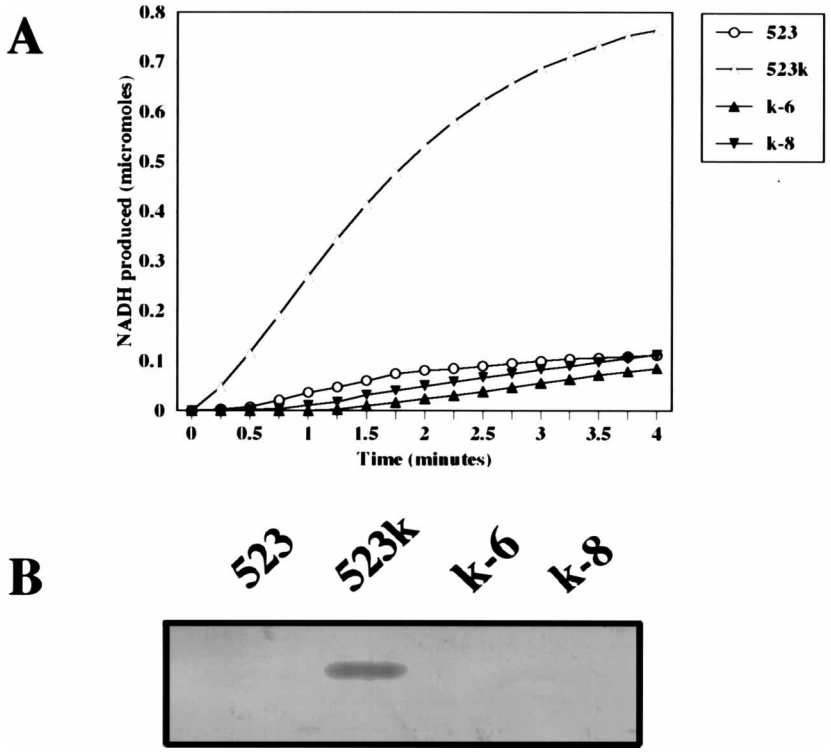

FIG. 5. Abolishment of D-nLDH activity and production in *ddh* inactivation mutants of 523k. (A) Kinetics of D-lactate-dependent NADH production in cytoplasmic fractions obtained from the indicated isolates. k-6, 523k-6; k-8, 523k-8. (B) Western blot of cytoplasmic fractions obtained from 523, 523k, 523k-6, and 523k-8 probed with the anti-D-nLDH antiserum.

related resistant strains. Therefore, no association was found between D-nLDH activity and vancomycin resistance in *S. aureus* or *S. haemolyticus*.

DISCUSSION

Our data indicate that overproduction of D-nLDH in the vancomycin-resistant *S. aureus* isolate 523k is not necessary for the glycopeptide resistance phenotype. Therefore, it is not surprising that we did not detect D-lactate-containing peptidoglycan precursors in that isolate. Thus, glycopeptide resistance in 523k occurs by a mechanism different from that in enterococci (3) and other intrinsically vancomycin-resistant gram-positive species, all of which synthesize peptidoglycan precursors that terminate in moieties other than D-Ala (9, 22).

Our data confirm previous speculation (30) that the 37-kDa protein found at an increased level in strain 523k is D-nLDH and is encoded by the *ddh* gene. Early studies of LDH activity in *S. aureus* demonstrated that both the L(+) and D(−) enantiomers of lactate are formed in *S. aureus* (17). However, since only one LDH enzyme was detected, it was concluded that D-lactate was not produced by D-nLDH, but rather by the coordinate activities of an NAD^+-dependent L-LDH (L-nLDH) and a lactate racemase that converted L-lactate to D-lactate. This view was called into question, however, by Götz and Schleifer (19), who revealed by zymogram analysis the presence of a unique D-nLDH enzyme in *S. aureus*. By demonstrating that the *ddh* gene produces D-nLDH activity in *E. coli* and that inactivating this gene in *S. aureus* abolishes D-nLDH activity, the genetic evidence is now provided to support the conclusion of Götz and Schleifer (19). However, from the low D-nLDH activity in the susceptible isolate, 523, and the high

TABLE 2. MICs for *ddh* inactivation mutants

Strain	MIC (μg/ml)[a]	
	VM	Tco
523	1	≤2
523k(pM952-2)	5	6
523k-14-6	5	6
523k-14-8	5	6

[a] Concentrations of glycopeptide tested were 0.5, 2.5, and 5.0 μg of vancomycin (VM)/ml and 2.0, 3.0, 4.0, 5.0, 6.0, 7.5, and 10.0 μg of teicoplanin (TCO)/ml.

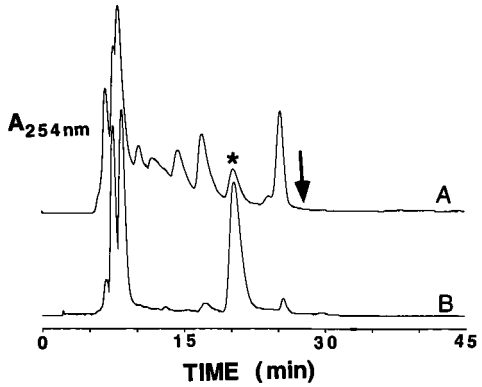

FIG. 6. HPLC chromatogram of peptidoglycan precursor fractions from isolates 523 (A) and 523k (B). Asterisk, peak corresponding to D-Ala-D-Ala-containing peptidoglycan precursors. Vertical arrow, lack of a peak at the retention time of the D-Ala-D-lactate-terminating precursor.

levels found in other clinical isolates (data not shown), we conclude that D-nLDH activity is variable among clinical isolates under the conditions tested.

Although D-nLDH production was not associated with glycopeptide resistance, the fact remains that some isolates obtained in the vancomycin selection protocol produced more or less D-nLDH than the susceptible parent strain; the reason for this is unclear. The increase in the abundance of *ddh* mRNA in isolate 523k implies the possible existence of a system for transcriptional regulation of the *ddh* gene in *S. aureus*. For example, *ddh* expression might be increased under anaerobic conditions, where the need for D-nLDH activity would be greatest. The monocistronic organization of the *ddh* gene rules out the idea that there could be a resistance-associated gene that is colinear with *ddh* and therefore coexpressed. However, it still remains possible that, at least in isolate 523k, *ddh* expression could be increased by factors that also increase the expression of a resistance-associated gene.

Proteins with molecular masses of 35, 37, and 39 kDa have also been observed at increased levels in several other glycopeptide-resistant staphylococcal clinical isolates (29, 35, 40) compared with levels in related or unrelated glycopeptide-susceptible isolates. However, it has been uncertain whether D-nLDH is structurally or functionally related to any of the proteins observed in these other glycopeptide-resistant staphylococcal isolates. By Western blotting and D-nLDH assay, we determined that the 35-kDa membrane protein produced by a teicoplanin-resistant *S. aureus* clinical isolate, 12873, (25, 40) is not D-nLDH (data not shown). An obvious corollary of this observation is that proteins with molecular masses similar to that of D-nLDH that are produced in increased amounts by glycopeptide-resistant isolates are not necessarily D-nLDH.

Considering what is known about the biosynthetic pathway used by enterococci to produce D-lactate-terminating precursors, it is not surprising that overproduction of D-nLDH in *S. aureus* did not result in production of D-lactate-terminating peptidoglycan precursors. The *van* gene clusters of enterococci with acquired glycopeptide resistance encode two enzyme activities besides VanH that are required for resistance: (i) a D-Ala-D-Ala ligase with broad substrate specificity, such as VanA or VanB (10, 14), which can efficiently synthesize D-Ala-D-lactate and (ii) a dipeptidase, VanX, which eliminates the pool of D-Ala-D-Ala dipeptides that would compete with D-Ala-D-lactate for incorporation into the precursor (38). These two enzyme activities have not been reported in *S. aureus*. However, the finding that D-lactate-terminating precursors were detected in *S. haemolyticus* isolate 18-88 and possibly also in isolate 18 (8), in which we found high D-nLDH activity, suggests that the D-Ala-D-Ala ligase in those strains is capable of synthesizing D-Ala-D-lactate, perhaps because of the high D-nLDH activity. If this is true, the small amount of D-lactate-terminating precursor in the isolates may be explained by an inefficient ligase activity, the lack of a D-Ala-D-Ala dipeptidase, or another undescribed enzyme activity. However, with respect to glycopeptide resistance, because evidence from the HPLC analysis suggested that D-lactate-terminating precursors could also be present in the susceptible *S. haemolyticus* parent isolate (8), it is unclear whether the depsipeptide precursors play a role in resistance in 18-88.

We speculate that the ability of some staphylococcal isolates to produce large amounts of D-nLDH could be the first of two or more steps leading to the ability of an isolate to produce a substantial pool of depsipeptide precursors which would produce vancomycin resistance. The second step might be a mutation that improves the efficiency of the apparatus involved in biosynthesis and/or stability of depsipeptide-containing peptidoglycan precursors. For example, under the selective pressure of glycopeptide therapy, a *Staphylococcus* strain which produces large amounts of D-nLDH could produce a chromosomal point mutation that broadens the substrate specificity of the D-Ala-D-Ala ligase, enabling it to efficiently produce the depsipeptide bond between D-Ala and D-lactate (36). Such a mutation, when combined with one that produces a VanX-like activity (perhaps in a gene encoding a carboxypeptidase), would produce a resistant strain capable of producing a substantial pool of depsipeptide-containing precursors. Either of these chromosomal mutations alone would decrease the amount of genetic material that would have to be acquired from the *van* gene clusters from enterococci to produce high-level vancomycin resistance.

We have recently directly shown by Western blotting that production of the high-molecular-weight penicillin-binding protein 2 (PBP2) is increased in all our laboratory-derived vancomycin- and teicoplanin-resistant *S. aureus* isolates compared with that in the susceptible parent clinical isolates (31). The production of PBP2 increased with serial incubation in increasing concentrations of vancomycin. Also, increased PBP2 production had a strong correlation with the MIC of vancomycin for the isolates. An increase in PBP2 production was also shown in teicoplanin-resistant clinical *S. aureus* isolates (29, 40) and more recently in a vancomycin-resistant clinical isolate from Japan (24). Moreover, the phenotypic and genotypic traits of the vancomycin-resistant *S. aureus* clinical isolate indicate that the mechanism of resistance is not due to the acquisition of vancomycin resistance genetic elements from enterococci (24). In contrast, the glycopeptide-resistant clinical isolates have intermediate levels of glycopeptide resistance, similar to those of the laboratory-derived glycopeptide-resistant isolates we have produced. Thus, the study of laboratory-derived isolates, in which isogenic parents are available for comparison, has proven to be an appropriate model system for identifying the resistance mechanism in this species. Therefore, PBP2, and possibly other PBPs, may play an important role in mediating vancomycin resistance in clinical isolates of *S. aureus*.

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REFERENCES

1. **Arthur, M., C. Molinas, and P. Courvalin.** 1992. The VanS-VanR two-component regulatory system controls synthesis of depsipeptide peptidoglycan precursors in *Enterococcus faecium* BM4147. J. Bacteriol. **174:**2583–2591.
2. **Arthur, M., C. Molinas, S. Dutka-Malen, and P. Courvalin.** 1991. Structural relationship between the vancomycin resistance protein VanH and 2-hydroxycarboxylic acid. Gene **103:**133–134.
3. **Arthur, M., P. Reynolds, and P. Courvalin.** 1996. Glycopeptide resistance in enterococci. Trends Microbiol. **4:**401–407.
4. **Augustin, J., and F. Götz.** 1990. Transformation of *Staphylococcus epidermidis* and other staphylococcal species with plasmid DNA by electroporation. FEMS Microbiol. Lett. **66:**203–208.
5. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1994. Current protocols in molecular biology, vol. 1, p. 1.8.4–1.8.5, 15.7.1.–15.7.3. John Wiley and Sons, Boston, Mass.
6. **Barna, J. C. J., and D. H. Williams.** 1984. The structure and mode of action of glycopeptide antibiotics of the vancomycin group. Annu. Rev. Microbiol. **38:**339–357.
7. **Biavasco, F., E. Giovanetti, M. P. Montanari, R. Lupidi, and P. E. Varaldo.** 1991. Development of in-vitro resistance to glycopeptide antibiotics: assess-

ment in staphylococci of different species. J. Antimicrob. Chemother. **27:**71–79.
8. **Billot-Klein, D., L. Gutmann, D. Bryant, D. Bell, J. van Heijenoort, J. Grewal, and D. M. Shlaes.** 1996. Peptidoglycan synthesis and structure in *Staphylococcus haemolyticus* expressing increasing levels of resistance to glycopeptide antibiotics. J. Bacteriol. **178:**4696–4703.
9. **Billot-Klein, D., L. Gutmann, S. Sablé, E. Guittet, and J. van Heijenoort.** 1994. Modification of peptidoglycan precursors is a common feature of the low-level vancomycin-resistant VANB-type enterococcus D366 and of the naturally glycopeptide-resistant species *Lactobacillus casei*, *Pediococcus pentosaceus*, *Leuconostoc mesenteroides*, and *Enterococcus gallinarum*. J. Bacteriol. **176:**2398–2405.
10. **Bugg, D. H., S. Dutka-Malen, M. Arthur, P. Courvalin, and C. T. Walsh.** 1991. Identification of vancomycin resistance protein VanA as a D-alanine:D-alanine ligase of altered substrate specificity. Biochemistry **30:**2017–2021.
11. **Bugg, T. D., G. D. Wright, S. Dutka-Malen, M. Arthur, P. Courvalin, and C. T. Walsh.** 1991. Molecular basis for vancomycin resistance in *Enterococcus faecium* BM4147: biosynthesis of a depsipeptide peptidoglycan precursor by vancomycin resistance proteins VanH and VanA. Biochemistry **30:**10408–10415.
12. **Bunch, P. K., F. Mat-Jan, N. A. Lee, B. A. DeAyala, and D. P. Clark.** 1995. Molecular cloning of the *ldhA* gene encoding the fermentative lactate dehydrogenase of *Escherichia coli*. GenBank Accession no. U36928.
12a. **Clark, D. P.** Personal communication.
13. **Daum, R. S., S. Gupta, R. Sabbagh, and W. M. Milewski.** 1992. Characterization of *Staphylococcus aureus* isolates with decreased susceptibility to vancomycin and teicoplanin: isolation and purification of a constitutively produced protein associated with decreased susceptibility. J. Infect. Dis. **166:**1066–1072.
14. **Evers, S., D. F. Sahm, and P. Courvalin.** 1993. The *vanB* gene of vancomycin resistant *Enterococcus faecalis* V583 is structurally related to D-Ala-D-Ala ligases and glycopeptide resistance proteins *vanA* and *vanC*. Gene **24:**143–144.
15. **Fairoz, M.-J., Y. A. Kiswar, and D. P. Clark.** 1989. Mutants of *Escherichia coli* deficient in fermentative lactate dehydrogenase. J. Bacteriol. **171:**342–348.
16. **Feinberg, A. P., and B. Vogelstein.** 1984. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **137:**266–267. (Addendum.)
17. **Garrard, W., and J. Lascelles.** 1968. Regulation of *Staphylococcus aureus* lactate dehydrogenase. J. Bacteriol. **95:**152–156.
18. **Garvie, E. I.** 1980. Bacterial lactate dehydrogenases. Microbiol. Rev. **44:**106–139.
19. **Götz, F., and K. H. Schleifer.** 1976. Comparative biochemistry of lactate dehydrogenases from staphylococci, p. 245–252. *In* J. Jeljaszewicz (ed.), Staphylococci and staphylococcal diseases. Gustav Fisher Verlag, Stuttgart, Germany.
20. **Greenwood, D., K. Bidgood, and M. Turner.** 1987. A comparison of the responses of staphylococci and streptococci to teicoplanin and vancomycin. J. Antimicrob. Chemother. **20:**155–164.
21. **Handwerger, S.** 1994. Alterations in peptidoglycan precursors and vancomycin susceptibility in Tn*917* insertion mutants of *Enterococcus faecalis* 221. Antimicrob. Agents Chemother. **38:**473–475.
22. **Handwerger, S., M. J. Pucci, K. J. Volk, J. Liu, and M. S. Lee.** 1994. Vancomycin-resistant *Leuconostoc mesenteroides* and *Lactobacillus casei* synthesize cytoplasmic precursors that terminate in lactate. J. Bacteriol. **176:**260–264.
23. **Herwaldt, L., L. Boykin, and M. Pfaller.** 1991. In vitro selection of resistance to vancomycin in bloodstream isolates of *Staphylococcus haemolyticus* and *Staphylococcus epidermidis*. Eur. J. Clin. Microbiol. Infect. Dis. **10:**1007–1012.
24. **Hiramatsu, K., H. Hanaki, T. Ino, K. Yabuta, T. Oguri, and F. C. Tenover.** 1997. Methicillin resistant *Staphylococcus aureus* clinical strain with reduced susceptibility. J. Antimicrob. Chemother. **40:**135–136.
25. **Kaatz, G. W., S. M. Seo, N. J. Dorman, and S. A. Lerner.** 1990. Emergence of teicoplanin resistance during therapy of *Staphylococcus aureus* endocarditis. J. Infect. Dis. **162:**103–108.
26. **Kreiswirth, B. N., S. Lofdahl, M. J. Betley, M. O'Reilly, P. M. Schlievert, M. S. Bergdoll, and R. P. Novick.** 1983. The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. Nature **305:**709–712.
27. **Krzysztof, S., and A. Tomasz.** 1996. A highly vancomycin resistant laboratory mutant of *Staphylococcus aureus*. FEMS Microbiol. Lett. **142:**161–166.
28. **Luchansky, J. B., A. K. Benson, and A. G. Atherly.** 1989. Construction, transfer and properties of a novel temperature-sensitive integrable plasmid for genomic analysis of *Staphylococcus aureus*. Mol. Microbiol. **3:**65–78.
29. **Mainardi, J. L., D. M. Shlaes, R. V. Goering, J. Shlaes, J. F. Acar, and F. W. Goldstein.** 1995. Decreased teicoplanin susceptibility of methicillin resistant strains of *Staphylococcus aureus*. J. Infect. Dis. **171:**1646–1650.
30. **Milewski, W. M., S. Boyle-Vavra, B. Moreira, C. Ebert, and R. Daum.** 1996. Overproduction of a 37-kDa cytoplasmic protein homologous to NAD^+-linked D-lactate dehydrogenase associated with vancomycin resistance in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **40:**166–172.
31. **Moreira, B., S. Boyle-Vavra, B. L. M. de Jonge, and R. S. Daum.** 1997. Increased production of penicillin-binding protein 2, increased detection of other penicillin-binding proteins, and decreased coagulase activity associated with glycopeptide resistance in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **41:**1788–1793.
32. **Muller, E., J. Hübner, N. Gutierrez, S. Takeda, D. A. Goldmann, and G. B. Pier.** 1993. Isolation and characterization of transposon mutants of *Staphylococcus epidermidis* deficient in capsular polysaccharide/adhesin and slime. Infect. Immun. **61:**551–558.
33. **National Committee for Clinical Laboratory Standards.** 1993. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7-A3. National Committee for Clinical Laboratory Standards, Villanova, Pa.
34. **Novick, R. N.** 1991. Genetic systems in staphylococci. Methods Enzymol. **204:**587–636.
35. **O'Hare, M. D., and P. Reynolds.** 1992. Novel membrane proteins present in teicoplanin-resistant, vancomycin sensitive, coagulase-negative staphylococcus spp. J. Antimicrob. Chemother. **30:**753–768.
36. **Park, I., C. Lin, and C. Walsh.** 1996. Gain of D-alanyl-D-lactate or D-lactyl-D-alanine synthetase activities in three active-site mutants of the *Escherichia coli* D-alanyl-D-alanine ligase B. Biochemistry **35:**10464–10471.
37. **Patti, J. M., T. Bremell, D. Krajewska-Pietrasik, A. Abdelnour, A. Tarkowski, C. Ryden, and M. Höök.** 1994. The *Staphylococcus aureus* collagen adhesin is a virulence determinant in experimental septic arthritis. Infect. Immun. **62:**152–161.
38. **Reynolds, P. E., F. Depardieu, S. Dutka-Malen, M. Arthur, and P. Courvalin.** 1994. Glycopeptide resistance mediated by enterococcal transposon Tn*1546* requires production of VanX for hydrolysis of D-alanyl-D-alanine. Mol. Microbiol. **13:**1065–1070.
39. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
40. **Shlaes, D. M., J. H. Shlaes, S. Vincent, L. Etter, P. D. Fey, and R. V. Goering.** 1993. Teicoplanin-resistant *Staphylococcus aureus* expresses a novel membrane protein and increases expression of penicillin-binding protein 2 complex. Antimicrob. Agents Chemother. **37:**2432–2437.
41. **Tarmy, E. M., and N. O. Kaplan.** 1959. Chemical characterization of D-lactate dehydrogenase from *Escherichia coli* B. J. Biol. Chem. **243:**2579–2586.
42. **Tarmy, E. M., and N. O. Kaplan.** 1968. Kinetics of *Escherichia coli* B D-lactate dehydrogenase and evidence for pyruvate controlled change in conformation. J. Biol. Chem. **243:**2587–2596.
43. **Youngman, P.** 1987. Plasmid vectors for recovering and exploiting Tn*917* transpositions in *Bacillus* and other Gram-positive bacteria, p. 79–103. *In* K. G. Hardy (ed.), Plasmids: a practical approach. IRL Press, Oxford, United Kingdom.
44. **Ziegler, D. W., R. N. Wolfe, and J. M. McGuire.** 1955–1956. Vancomycin, a new antibiotic. II. In vitro antibacterial studies. Antibiotics Annu. **1955–1956:**612–618.