ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 1996, p. 166–172 Vol. 40, No. 1
0066-4804/96/$04.00+0

Overproduction of a 37-Kilodalton Cytoplasmic Protein Homologous to NAD$^+$-Linked D-Lactate Dehydrogenase Associated with Vancomycin Resistance in *Staphylococcus aureus*

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Received 24 July 1995/Returned for modification 29 August 1995/Accepted 1 November 1995

We previously reported the isolation of a laboratory-derived *Staphylococcus aureus* mutant, 523k, that has constitutive low-level resistance to vancomycin (MIC = 5 μg/ml) and teicoplanin (MIC = 5 μg/ml) and elaborates a ca. 39-kDa cytoplasmic protein that was not detected in the parent strain 523 (MIC = 1 μg/ml). We have now detected the protein in strain 523 by immunoblotting with antiserum raised against the protein. Consistent with our initial observations, densitometric analysis of the immunoblots revealed an increased production of the protein in 523k compared with that of the susceptible parent 523. The 5′ region of the gene encoding the protein of interest was identified by nucleotide sequencing a PCR product amplified from the genome of 523k with degenerate primers designed to encode the amino acid sequence of proteolytic peptides obtained from the protein. The remainder of the gene was identified by library screening, PCR, and nucleotide sequencing. The gene encodes a 36.7-kDa protein with homology to a family of bacterial NAD$^+$-dependent, D-specific 2-hydroxyacid dehydrogenases which includes both D-lactate dehydrogenase and the enterococcal vancomycin resistance protein VanH and is therefore designated *ddh*. Increased production of the product of *ddh*, Ddh, was associated with increased D-lactate dehydrogenase activity in 523k, a finding which suggested that Ddh is likely to be the D-lactate dehydrogenase previously identified in *S. aureus*. The increased D-lactate dehydrogenase activity in strain 523k and the structural similarities among Ddh, D-lactate dehydrogenase, and VanH suggest that overproduction of Ddh might play a role in vancomycin resistance in this strain.

The isolation of clinical strains of staphylococci resistant (MIC ≥ 32 μg/ml) (37) to glycopeptide antimicrobial agents such as vancomycin or teicoplanin has been infrequent and confined to two species of coagulase-negative staphylococci, *Staphylococcus haemolyticus* and *Staphylococcus epidermidis* (3, 6, 17, 40, 44, 46). In addition, several investigators have isolated *Staphylococcus aureus* (28, 35) and coagulase-negative staphylococci (13, 14, 16, 18, 22, 45, 48, 54, 55, 59) for which the MICs are beyond the susceptibility breakpoints (MIC > 4 μg/ml for vancomycin and MIC > 8 μg/ml for teicoplanin) (37) of these compounds. However, the genetic and biochemical bases of glycopeptide resistance in this genus remain uncharacterized. Because the clinical use of vancomycin has increased greatly in the United States, it has been suggested (50) that resistance to this compound among clinical isolates of *S. aureus* will soon be a concern. The recent demonstration that genes effecting high-level resistance can be transferred from *Enterococcus faecalis* to *S. aureus* in vitro (38) makes this prediction plausible.

In the laboratory, we (15) and others (10, 20, 24, 60) have shown that *S. aureus* strains for which the MICs are beyond the susceptibility breakpoint of vancomycin or teicoplanin can be isolated during passage in media containing these glycopeptides. An investigation of the mechanisms of resistance in such laboratory-derived mutants might provide clues for resistance strategies that occur in vivo.

We previously reported the isolation of a mutant strain, 523k, with decreased susceptibilities to vancomycin and teicoplanin by incubating clinical isolate 523 in vancomycin (15). The resistant phenotype was constitutive and associated with the production of a protein which was estimated to have a molecular mass of 39 kDa. Others (40, 51) also demonstrated de novo or increased production of proteins with molecular masses of ca. 39 or 35 kDa in clinical isolates and laboratory-derived staphylococci for which the MICs are beyond the susceptibility breakpoint. However, the identities of these proteins or the roles they play in resistance have not been determined.

The aims of this study were to identify the ca. 39-kDa protein that is overproduced in strain 523k by identifying and sequencing its gene and to characterize the production of the protein in strains 523 and 523k.

(The results of this study were presented in part at the 35th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Diego, Calif., September 1995.)

MATERIALS AND METHODS

Growth conditions, bacteria, and bacteriophage. Conditions for growth, maintenance, and storage of *S. aureus* isolates were previously described (15). Isolates 523 (MICs = 1.25 and 0.625 μg/ml for vancomycin and teicoplanin, respectively) and 523k (MIC = 5 μg/ml for vancomycin and for teicoplanin) were previously described (15). Strain FDA485 was a gift from Clontech (Palo Alto, Calif.), and RN4220 (32) was provided by Jean Lee (Boston, Mass.).

Escherichia coli isolates were grown in Luria-Bertani broth (Gibco BRL, Grand Island, N.Y.) or Luria-Bertani agar (Gibco BRL) and stored at −70°C in

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skim milk (Difco, Detroit, Mich.). Where appropriate, ampicillin (Sigma, St. Louis, Mo.) at a concentration of 100 μg/ml, IPTG (isopropyl-β-D-thiogalactopyranoside; Gibco BRL) at 0.5 mM, and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Gibco BRL) at 40 μg/ml were added to the media. *E. coli* Y1090 obtained from Clontech was used as the host for library screening, and Electro-maxDH 10-BTH and DH5α obtained from Gibco BRL were used as transformation recipients in cloning plasmid DNA.

The λgt11 library containing genomic DNA from *S. aureus* FDA485 was obtained from Clontech and had been produced by mechanical shearing of genomic DNA followed by ligation with *Eco*RI linkers. Procedures for propagating and screening phage DNA were recommended by Clontech.

Purification of the ca. 39-kDa protein. Preparation and separation of cell fractions from 523 and 523k and the purification of the ca. 39-kDa protein from 523k was accomplished by sonication, differential centrifugation, DEAE column chromatography, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroelution as previously described (15).

Preparation of polyclonal antibody, immunoblotting, and densitometry. New Zealand rabbits were primed with 170 μg of purified protein (15) mixed 1:1 with complete Freund's adjuvant (Sigma). Two booster doses, each consisting of 45 μg of protein mixed 1:1 with incomplete Freund's adjuvant, were given at 1-month intervals. The highest titer of antibody directed against the protein was detected by an enzyme-linked immunosorbent assay in serum obtained after the second booster dose.

For immunoblotting, cytoplasmic and membrane fractions from 523 and 523k were obtained by differential centrifugation of cell lysates produced by sonication of overnight cultures as previously described (15) except that the initial low-speed spin at 5,900 × *g* was performed twice to prevent contamination of the membrane fraction by unlysed cells. Protein concentrations in the lysates were determined by the Lowry method modified by Peterson (41). Equal amounts of protein from each lysate were separated by SDS–12% PAGE (33) and electroblotted onto nitrocellulose as previously described (43). Membranes were incubated in a 1.6×10^{-5} dilution of rabbit antiserum raised against the ca. 39-kDa protein. The secondary antibody (goat anti-rabbit immunoglobulin G conjugated with alkaline phosphatase; Sigma) was used at a dilution of 10^{-3}.

Immunodetection was performed with BCIP (5-bromo-4-chloro-3-indolylphosphate toluidinium; Sigma) and nitroblue tetrazolium (Sigma) according to the manufacturer's instructions. For densitometry, membranes were scanned by reflected light with an OmniMedia x-ray scanner model 12cx (Lumisys, Sunnyvale, Calif.). Quantitation of band intensities was performed with NIH Image software.

Amino acid sequencing. Amino acid sequencing was performed by Edman degradation with an Applied Biosystems pulsed-liquid sequencer model 473A (Applied Biosystems, Foster City, Calif.) at the protein sequencing core facility at the University of Chicago. For determining the N-terminal amino acid sequence, the purified protein was subjected to SDS–12% PAGE (33) and electroblotted onto polyvinylidene difluoride membrane (Applied Biosystems).

Peptide fragments were prepared by the removal of SDS from the purified, electroeluted protein according to the solvent extraction-precipitation method of Wessel and Flügge (57). The pellet required 8 M urea in 50 mM Tris-HCl (pH 8.0) for suspension and was subsequently diluted in the same buffer to a final concentration of 2 M urea. The protein was incubated in 0.75 U of endopeptidase Lys C (Boehringer-Mannheim Corp., Indianapolis, Ind.) at 37°C for 24 h and then precipitated in 0.5% trifluoroacetic acid and centrifuged. Peptide fragments in the supernatant were separated by reversed-phase high-performance liquid chromatography (HPLC) with an Altex C-18 Ion Pair column (Perkin-Elmer Cetus, Norwalk, Conn.), and four peptides designated 5, 6, 12, and 20 were sequenced by Edman degradation.

Reagents for analysis of DNA. Cloning of DNA fragments was performed by standard procedures (43). Radionucleotides were purchased from Amersham (Arlington Heights, Ill.). Oligonucleotide primers were synthesized on an Applied Biosystems DNA synthesizer, model 380B, at the Howard Hughes Medical Institute at the University of Chicago. Small-scale plasmid preparations were performed with the Flexi-Prep kit (Pharmacia Biotech, Piscataway, N.J.).

Preparation of *S. aureus* DNA. Total cell DNA was prepared from *S. aureus* isolates by the procedure described by Matthews et al. (36), except that the lysostaphin concentrations were 50 μg/ml for strains 523, FDA485, and RN4220 and 1,000 μg/ml for 523k.

Conditions for performing PCR. All PCR mixtures consisted of a 50-μl final reaction volume containing genomic DNA (50 ng) prepared from *S. aureus*, 100 μM each deoxynucleoside triphosphate, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.001% gelatin, 1.25 U of *AmpliTaq* DNA polymerase (Perkin-Elmer Cetus), and the indicated concentration of primers (see below).

To produce the 952-bp amplimer from 523k containing the partial gene encoding the ca. 39-kDa protein, degenerate oligonucleotide primers 1 [5′-TA(T/C) CA(T/C) TT(A/G) TT(T/C) GA(T/C) AAA-3′] and 2 [5′-(T/C)TT (A/T/G/C)AC (A/G)TG (A/G)TC (A/G)AA CAT (A/T/G/C)GC-3′] were designed to encode, respectively, the sense strand of peptide 6 and the antisense strand of peptide 5; these primers were used at a concentration of 200 pM each, and the PCR profile consisted of 60 s of denaturation at 94°C, 90 s of annealing at 50°C, and 90 s of extension at 70°C performed for 40 cycles.

To produce the 1,431-bp amplimer containing the full-length gene of interest from strain 523k, primers 3 (5′-AGA CTA AAG TAA CCC GTT TAG AAT-3′) and 4 (5′-AAG CGG CTA TCA TGA TAC TAA ATA-3′) (Fig. 1), designed to hybridize to sequences flanking the 5′ and 3′ termini of the complete open reading frame (ORF), respectively, were used at a concentration of 100 pM each. The PCR profile consisted of 60 s of denaturation at 94°C, 90 s of annealing at 56°C, and 90 s of extension at 72°C performed for 35 cycles.

Nucleotide sequencing. The 952-bp PCR product from strain 523k was cloned into the *Eco*RV site of the pBluescriptII SK$^+$ vector (Stratagene, La Jolla, Calif.) to produce pM952, and the 4-kb FD485 library fragment was cloned into the *Eco*RI site of pBluescriptII SK$^+$ to produce pM4000. For the sequencing of plasmids, DNA was denatured with alkali and sequenced with the Sequenase 2.0 DNA sequencing kit (United States Biochemical Corp., Cleveland, Ohio) with [α-^{35}S]dATP. The 1,431-bp PCR product from 523k was purified (GlassMax DNA Isolation Spin Cartridge System; Gibco BRL) and sequenced by the *fmol* DNA sequencing system (Promega, Madison, Wis.). Residues 1 to 933 were determined for both strands of the pM952 insert by using a gene walking strategy starting with T3 and T7 promoter primers (Stratagene) and ending with various internal primers. Residues 934 to 1480 were determined from the 1,431-bp PCR product, starting with a primer which overlapped the 952-bp PCR product.

Probe labeling. To prepare the probe for screening of the FDA485 λgt11 library and analysis of Southern blots, the 952-bp *Eco*RI-*Hin*dIII insert from pM952 was purified by agarose gel electrophoresis and the GlassMax DNA Isolation Spin Cartridge System. Random primer labeling of the probe was performed with the Rad Prime Labeling System (Gibco BRL) with [α-^{32}P]dCTP, and unincorporated nucleotides were removed with a Bio-Spin 6 chromatography column (Bio-Rad, Richmond, Calif.).

D-(−)-Lactate dehydrogenase assays. NAD$^+$-linked, D-lactate dehydrogenase activity was determined by measuring NAD$^+$ reduction in the presence of D-lactate. Cytoplasmic extracts (prepared from overnight cultures as described above) were added to an assay mixture containing 100 mM Tris (pH 8.0), 4.8 mM KCN, and 10 mM NAD$^+$ (all from Sigma) and equilibrated for 1 min at 25°C. Lithium D-lactate (400 mM) (Boehringer-Mannheim) was added to a final volume of 1 ml, and NADH production was measured as an increase in the A_{340}. Units were calculated as micromoles of NADH produced per minute per microgram of protein after subtracting the rate of NADH production in the absence of D-lactate. Protein concentrations were determined by the Bio-Rad protein assay.

Computer analysis. DNA sequences were analyzed with the DNA Strider 2.0 sequence analysis software or Mac Vector 4.1 (Eastman Kodak, Rochester, N.Y.). The National Center for Biotechnology Information database was screened with the Basic Local Alignment Sequencing Tool (BLAST) (2). Protein sequence alignments were performed with the Clustal W (version 1.4) multiple-alignment program (53).

Nucleotide sequence accession number. The nucleotide sequence of the *S. aureus ddh* gene was submitted to the GenBank database under accession number U31175.

RESULTS

Identification of the gene encoding the ca. 39-kDa protein in strain 523k. The N-terminal sequence of the purified ca. 39-kDa protein was determined with ambiguities (marked in italic type) at positions 9 and 19 (TKIMFFGT[*C* or *W*]DYEKEMALN*N*GKKNNVE). In addition, the sequences of four peptides produced by endopeptidase Lys C digestion of the purified protein were peptide 5 (AMFDHVK), peptide 6 (ESYHLFDK), peptide 12 (DADIISLHVPANK), and peptide 20 (*S*IAQ[X]TAGF*D*MYDLDLAK*P*); ambiguities at positions 1, 10, and 19 are indicated with italic type.

To identify the gene encoding this protein, the peptide sequences were used to design degenerate oligonucleotide primers for PCR. With a sense primer corresponding to peptide 6 (primer 1) and an antisense primer corresponding to peptide 5 (primer 2), PCR with whole-cell DNA from strain 523k produced four fragments which were cloned and sequenced. One of these, a 952-bp fragment (in plasmid pM952), contained a partial ORF (ORF′) which encoded the N-terminal sequence of the protein and the proteolytic peptides 5, 6, 12, and 20 reported above (Fig. 1). The other three fragments contained unrelated sequences.

Curiously, the 952-bp fragment was flanked at each end by primer 2-specific sequences. The primer 2-specific sequence at the 3′ terminus of the ORF′ was a perfect match, but the primer 2-specific sequence found upstream of the ORF′ was imperfect and most likely resulted from nonspecific priming during PCR. This was fortuitous since peptides 5 and 6 en-

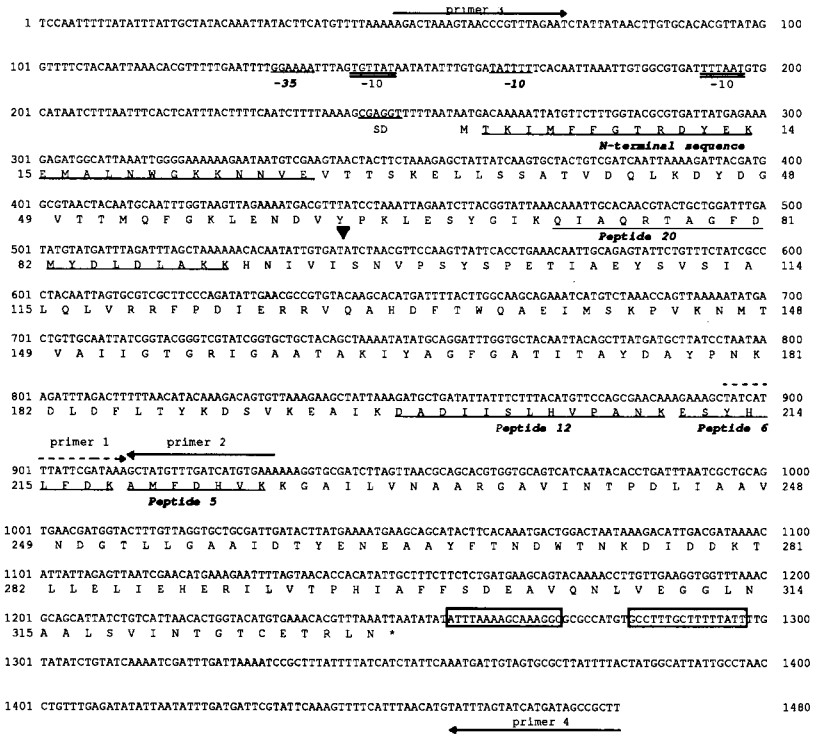

FIG. 1. Nucleotide and deduced amino acid sequences of the *S. aureus* gene encoding the protein produced at increased levels in strain 523k. Nucleotides 1 to 933 were determined on both strands of the 952-bp PCR fragment obtained from strain 523k. Nucleotides 934 to 1480 were determined from the 1,431-bp PCR product from strain 523k. Amino acid residues are designated by a single-letter code below the nucleotide sequence. The putative promoter sequences (−35, −10), Shine-Dalgarno sequence (SD), transcriptional terminator (boxes), and stop codon (asterisk) are indicated. Additional putative −10 sequences identical to those found in other staphylococcal genes (39) are indicated by a double line beneath the sequence. The *Eco*RV cleavage site is marked with a vertical arrowhead. The 26-amino-acid N-terminal sequence and peptide sequences 5, 6, 12, and 20 encoded by the ORF are indicated. Primer annealing sites and their orientation are indicated by horizontal arrows, with the exception of the dotted line designating primer 1.

coded, respectively, by primers 1 and 2 are encoded sequentially in the ORF′ (Fig. 1) and therefore would produce a nondetectable product. The N-terminal sequence in the 952-bp fragment started 262 bp downstream from the end of the upstream primer 2 sequence. The ORF′ ended at the terminus of the 952-bp fragment and lacked a stop codon and about 300 bp of additional coding sequence that would be needed for a gene to encode a ca. 39-kDa protein.

Southern blotting analysis of total cellular DNA obtained from 523 and 523k with the use of a probe prepared from the 952-bp insert from pM952 revealed that both strains have an identically sized copy of the *ddh* locus contained on a 3.5-kb *Hin*dIII fragment (Fig. 2A). Two unrelated strains of *S. aureus*, FDA485 and RN4220, each also have a copy of the gene (Fig. 2B), as indicated by two hybridizing fragments produced after digestion at the internal *Eco*RV site.

To obtain the 3′ sequence of the gene that was not contained in the 952-bp amplimer, a 4-kb fragment from a λgt11 library of *S. aureus* FDA485 which hybridized with the pM952 insert was cloned and sequenced. This 4-kb fragment encoded sequences which overlapped the 3′ end of the pM952 ORF′ and contained the additional 3′ sequence that was not present in the 952-bp PCR product. Restriction analysis indicated that this 4-kb FDA485 fragment lacked a portion of the 5′ region of the ORF′.

The full-length gene was identified in strain 523k by PCR performed with a forward primer (primer 3) designed against the 5′ noncoding region flanking the ORF′ from pM952 and a reverse primer (primer 4) designed against the 3′ noncoding region flanking the ORF′ from the FDA485 insert (Fig. 1). The resultant 1,431-bp PCR product obtained from strain 523k was then used as the template to complete the nucleotide sequence of the 3′ region (corresponding to nucleotides 934 to 1480 in Fig. 1) of the gene in this strain. When the portion of the sequence of the gene that was obtained from strain FDA485

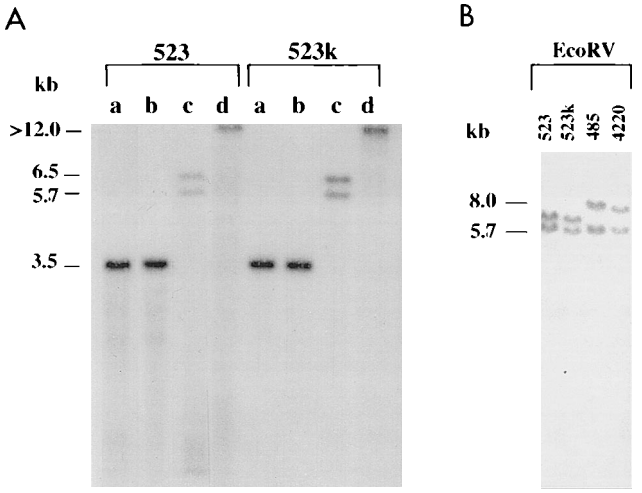

FIG. 2. Southern blotting with the ^{32}P-labeled 952-bp insert from pM952. (A) Whole-cell DNA from strains 523 and 523k digested with (lanes a) *Hin*dIII, (lanes b) *Hin*dIII plus *Eco*RI, (lanes c) *Eco*RV, and (lanes d) *Eco*RI. (B) Chromosomal DNA digested with *Eco*RV from strains 523, 523k, FDA485 (485), and RN4220 (4220).

was compared with the corresponding region of the gene from strain 523k (nucleotides 934 to 1480 in Fig. 1), the few differences noted at the nucleotide level did not result in any differences in the deduced amino acid sequences.

Nucleotide sequence analysis. The complete nucleotide sequence of the gene encoding the overproduced protein in strain 523k is shown in Fig. 1. A 990-bp ORF encodes a protein of 330 amino acids with a size of 36,679 Da, close to the size of 39 kDa estimated by SDS-PAGE. From the deduced amino acid composition of the translated ORF, the protein is predicted to have a pI of 4.93, similar to our own measurements for this protein (data not shown). The 34% G + C content of the ORF is typical of the *S. aureus* genome (39). While no mismatches were found between the deduced amino acid sequence and sequences determined from peptides 5, 6, 12, and 20, the nucleotide sequence clarified the few ambiguities in the sequences of the N terminus and peptide 20.

Two ATG start codons could potentially be associated with this ORF: one at nucleotide position 196 and the other at nucleotide position 259 (Fig. 1). Although the 21-residue amino acid sequence following the first ATG at nucleotide 196 is slightly hydrophobic, it does not match to the −3, −1 consensus of von Heijne (56) predicted for a typical export signal sequence. Thus, we assigned the start codon to the second ATG codon at nucleotide 259, in close proximity to a putative ribosome-binding site, CGAGGT (Fig. 1). The predicted N-terminal sequence agrees with that determined from the protein, except for the absence of the methionine encoded by the putative initiation codon which was likely posttranslationally cleaved.

Upstream from the putative ATG translational start codon are sequence elements identical to −10 and −35 promoter sequences that have been identified in other staphylococcal genes (39). An inverted-repeat sequence occurs between nucleotides 1257 and 1297, 6 bp downstream from the putative translational stop codon (TAA), which could form a stem-loop structure and function as a factor-independent transcription termination signal.

Amino acid sequence homology with a family of D-2-hydroxyacid dehydrogenases. When the gene bank was searched at the nucleotide level, no matches were found which had significant homology with the sequence shown in Fig. 1. However, when the protein sequence database was searched, significant homology was found between the deduced amino sequence shown in Fig. 1 and proteins from a family of D-2-hydroxyacid dehydrogenases (5, 8, 9, 23, 30, 52). Included in this family are NAD^+-linked D-lactate dehydrogenases (D-nLDH according to the nomenclature of Garvie [19]) from several gram-positive, lactic acid-producing species (Fig. 3). Importantly, also included is VanH (5), a protein necessary for high-level vancomycin resistance in the VanA class of vancomycin-resistant enterococci (4). In a multiple-sequence alignment (Fig. 3) between the putative *S. aureus* dehydrogenase and members of the D-2-hydroxyacid dehydrogenase family, the characteristic NAD^+ cofactor binding motif, GXGXX $G(X_{17-20})D$ (X is any amino acid) (47, 58) is conserved with respect to sequence and position. In the putative staphylococcal dehydrogenase, the last residue in this domain is Asp, which is more like D-nLDH than VanH and indicates a preference for NADH rather than NADP as the cofactor (5, 49). Importantly, a number of other conserved residues associated with cofactor or substrate binding (30, 31, 52) by members of this family also occur in expected positions in the amino acid sequence of the *S. aureus* protein. These include Arg 235; Asn 232; Asp 84, 200, and 259; Glu 108 and 264; Gly 79; His 296; Lys 225; Pro 99; and Tyr 101. Because of these relationships, we have designated the gene found in *S. aureus ddh* for a putative NAD^+-dependent D-specific 2-hydroxyacid dehydrogenase.

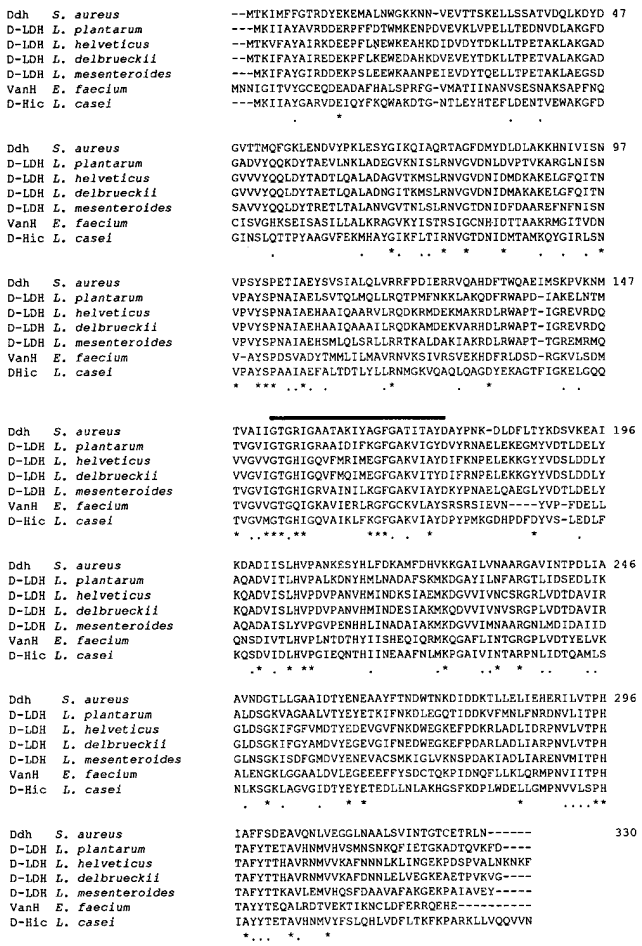

FIG. 3. Multiple alignment among the sequences of the *S. aureus* Ddh protein from 523k (first line) and various enzymes in the D-2-hydroxyacid dehydrogenase family (D-nLDH from *Lactobacillus plantarum* [52], *Lactobacillus helveticus* [29], *Lactobacillus delbrueckii* subsp. *bulgaricus* [8], and *Leuconostoc mesenteroides* [accession number L29327], VanH from *E. faecium* [5], and D-Hic from *Lactobacillus casei* [34]). Asterisks indicate identical residues and dots indicate conservative replacements. The overlying bar indicates the conserved NAD^+-binding domain $GXGXXG(X_{17-20})$-D (X is any amino acid) (47, 58).

Individual sequence alignments reveal that of all of the members of the D-2-hydroxyacid dehydrogenase family, the *S. aureus* protein has the most similarity with D-nLDH from *Lactobacillus plantarum* (61% similarity and 40% identity). As examples of relatedness between the *S. aureus* dehydrogenase and other family members, the percentages of residues that are similar or identical, respectively, are 57 and 36% for D-hydroxyisocaproate dehydrogenase (DHic) of *Lactobacillus delbrueckii* subsp. *bulgaricus* (9), 49 and 33% for D-nLDH from *Leuconostoc mesenteroides* (accession number L29327), and 52 and 28% for VanH from *Enterococcus faecium* (5).

Increased D-nLDH activity in strain 523k associated with increased production of the Ddh protein. Prior examination of SDS-polyacrylamide gels of cytoplasmic fractions from strains 523 and 523k revealed that a band with a molecular mass close to the 37-kDa mass predicted from the sequence of Ddh was readily detected in resistant strain 523k but not in parent iso-

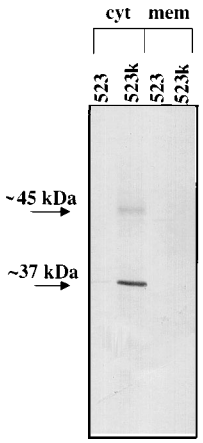

FIG. 4. Western immunoblot of the cytoplasmic (cyt) and membrane (mem) fractions from strains 523 and 523k probed with polyclonal rabbit antiserum raised against the Ddh protein purified from strain 523k.

late 523 (15). To clarify differences in production of Ddh between strains 523 and 523k, the rabbit polyclonal antiserum raised against the purified protein was used to probe immunoblots of cytoplasmic and membrane fractions from these strains. A band of about 37 kDa reacted with the antiserum in cytoplasmic fractions of both isolates with 10- to 17-fold greater reactivity in the band from strain 523k (Fig. 4), as determined by scanning densitometry. The 37-kDa band was not detected with the antiserum in the membrane fractions of either strain, which demonstrated that Ddh is localized in the cytoplasm. The preimmune serum used as the primary antibody or the secondary antibody used alone did not react with the band (data not shown). The ca. 45-kDa band seen in immunoblots of the cytoplasmic fraction of strain 523k reacted nonspecifically with the anti-Ddh antiserum as indicated by its reactivity with preimmune serum.

Because of the increased production of Ddh in the cytoplasm of strain 523k (Fig. 4) and the homology found with D-nLDH from *Lactobacillus plantarum*, D-nLDH activities in cytoplasmic extracts were compared between strains 523 and 523k. The results of four separate assays (in duplicate) indicated that isolate 523k had a mean eightfold (with a standard error of the mean of ± threefold) greater level of specific activity than that of parent isolate 523.

DISCUSSION

We identified the gene which encodes a 36.7-kDa protein that is produced at increased levels in a laboratory-derived vancomycin-resistant mutant of *S. aureus* over those of its parent strain. We designated this gene *ddh* for a D-specific 2-hydroxyacid dehydrogenase since the deduced amino acid sequence is 28 to 40% identical and 49 to 61% similar to those of members of the D-2-hydroxyacid dehydrogenase family, with identity occurring in many residues considered important for enzyme function. VanH and several D-nLDHs are members of this family and have been shown to be structurally and functionally similar by their ability to convert pyruvate to D-lactate (12). VanH plays an essential role in vancomycin resistance in resistant *E. faecium* and *E. faecalis* (4) (see below). It is therefore noteworthy that the increased production of Ddh in the vancomycin-resistant staphylococcal strain 523k was associated with an increase in D-nLDH activity, an observation suggesting that this protein might play a role in the resistance.

Vancomycin acts by binding the dipeptide, D-alanyl-D-alanine (D-Ala-D-Ala), at the carboxy terminus of the stem pentapeptide of muramyl peptidoglycan precursors (7). Such precursors bound to vancomycin are presumed to be inhibited from transglycosidation and transpeptidation (7), reactions required for peptidoglycan synthesis. The role that VanH plays in vancomycin resistance in resistant enterococci is to produce D-lactate (12), which is incorporated as the terminal residue of peptidoglycan precursors (1, 12, 25, 27). Such D-Ala-D-lactate-containing precursors have 1,000-fold decreased levels of affinity for vancomycin relative to D-Ala-D-Ala-containing precursors (12), a change which is thought to explain resistance to vancomycin.

The *S. aureus* D-nLDH enzyme was previously characterized biochemically (21). However, the gene encoding this protein was not identified. We propose that D-nLDH is encoded by the *ddh* gene on the basis of the following: (i) the highest degree of similarity was found with D-nLDH from *Lactobacillus plantarum* (61% similarity, 40% identity); (ii) the predicted 36.7-kDa molecular mass of the Ddh protein is compatible with the monomer size required to form a homodimer of ca. 70 kDa, the size of *S. aureus* D-nLDH estimated by gel filtration (21); and (iii) increased production of the *ddh* gene product in strain 523k was associated with increased D-nLDH activity. Thus, if Ddh is D-nLDH and increased production of the enzyme in strain 523k is related to vancomycin resistance, it would appear that a common metabolic protein has been exploited in association with survival in the presence of this antimicrobial agent. Moreover, one might speculate that the increase in the level of D-nLDH activity in 523k may result in the production of peptidoglycan precursors that terminate in D-lactate as is the case for species of gram-positive, lactic acid bacteria from the genera *Leuconostoc*, *Lactobacillus*, and *Pediococcus* which have intrinsic high-level resistance to glycopeptides (42) and produce D-lactate as the end product of their carbon dissimilation pathway (19). In certain strains from these genera the presence of peptidoglycan precursors with stem pentapeptides terminating in D-lactate has been demonstrated (11, 26).

Others have found novel or increased levels of protein production in glycopeptide-resistant staphylococci. For example, the production of membrane proteins with molecular masses of 35 and 39 kDa, comparable to the mass of Ddh, were associated with teicoplanin resistance in *S. aureus* and coagulase-negative staphylococci (35, 40, 51). Because these proteins were found predominantly in membrane fractions, they are not likely to be the Ddh protein.

Whether increased production of Ddh in strain 523k is due to changes in *ddh* gene expression is as yet unknown. Increased copy number is an unlikely explanation as evidenced by Southern blotting with the *ddh* probe. Other possible explanations are being pursued.

In addition to increased production of D-nLDH, 523k possesses pleiotropic cell surface-associated changes, including increased resistance to lysostaphin, the loss of both capsule and phage typeability, altered coagulase activity, and increased cell wall thickening (15). Therefore, it is possible that the development of resistance to glycopeptides in *S. aureus* involves multiple steps, one of which may involve an increased level of production of the VanH-like protein we have identified here.

ACKNOWLEDGMENTS

We are grateful to the late Howard Tager, who performed the endopeptidase Lys C digestion and the reversed-phase HPLC on the peptide fragments, Giri Reddy, who performed the amino acid sequence analysis, and Wolfgang Epstein, who provided encouragement

and a careful review of the manuscript. Thanks also go to Steve Duguay, Lucia Rothman-Denes, Piotr Gornicki, and Joseph LaMendola, who provided thoughtful discussion and technical advice.

Support for this research was provided in part by the Blowitz-Ridgeway Foundation, Des Plaines, Ill., and the Children's Research Foundation, Chicago, Ill.

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