JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 2005, p. 179–185 Vol. 43, No. 1
0095-1137/05/$08.00+0 doi:10.1128/JCM.43.1.179–185.2005

First Report of Vancomycin-Resistant Staphylococci Isolated from Healthy Carriers in Brazil

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Received 29 July 2004/Returned for modification 25 August 2004/Accepted 13 September 2004

Reduced susceptibility or resistance to vancomycin has been reported among clinical isolates of staphylococci in previous studies. In the present study we report on the isolation of four vancomycin-resistant staphylococcal strains from healthy carriers inside and outside the hospital environment. These carriers did not receive treatment with any antibiotic. All coagulase-negative staphylococcal strains showed variable levels of resistance to several antimicrobial agents, including oxacillin, and unstable resistance to vancomycin, with decreased vancomycin MICs (<4 mg/liter) after 10 days of passage in a nonselective medium. However, exposure of these revertants to vancomycin selected staphylococcal strains resistant to vancomycin at very high frequencies (10^{-2} and 10^{-3}). The vancomycin resistance in these staphylococcal strains was not mediated by the *van* gene. The cell wall of the staphylococcal strains studied became thickest after culture in medium containing vancomycin, and the differences in cell wall thickness were statistically significant ($P < 0.001$). Thus, the thickening of the cell wall in these staphylococcal strains may be an important contributor to vancomycin resistance.

Coagulase-negative staphylococci (CoNS) and, occasionally, *Staphylococcus aureus* are part of the microbiota found at various sites in the human body (skin and mucosae) and may serve as sources of infection when the normal defenses of the host organism are impaired by associated diseases (e.g., viral diseases), immunosuppressive therapy, and the use of invasive devices or when the delicate balance of this microbiota is altered by antimicrobial therapy. Staphylococci have a remarkable ability to adapt rapidly to antibiotic pressure (10, 16).

The antibiotic vancomycin is useful against gram-positive pathogens. However, with its increased use, resistance has been noticed in various species of bacteria, mainly enterococci. Vancomycin-resistant *Staphylococcus aureus* (VRSA) and *S. aureus* strains with reduced susceptibilities to vancomycin have been isolated in several countries (2, 12, 19, 20, 28). Coagulase-negative staphylococci with heteroresistance to vancomycin have also been reported (4, 29). The term heteroresistance to vancomycin has been used for staphylococcal isolates that contain a cell population with different levels of vancomycin susceptibility, including vancomycin-resistant cells.

The term vancomycin-resistant staphylococci (VRS) as defined in this study is based on the vancomycin breakpoint of the British Society for Antimicrobial Chemotherapy, according to which a strain for which the MIC is 8 μg/ml is defined as resistant. The NCCLS (18) has suggested that all staphylococci for which the vancomycin MIC is ≥4 μg/ml should be analyzed more carefully.

The genetic basis for vancomycin resistance in staphylococci has not been elucidated. Cell wall thickening is considered a prerequisite for vancomycin resistance (7, 8, 12). However, a decrease in peptidoglycan cross-linkage and a high content of free D-alanyl–D-alanine residues in the cell wall may increase the resistance of the strain (7, 21, 23). The *van* genes involved in glycopeptide resistance in enterococci have not been associated with resistance to vancomycin in staphylococci, but three VRSA strains harboring the *vanA* gene were isolated in Michigan, New York, and Pennsylvania (5, 6, 27).

The isolation of *S. aureus* and CoNS strains exhibiting reduced susceptibility or resistance to vancomycin from clinical samples from patients who had received glycopeptide antibiotics or any other course of antimicrobial agents has been described previously (20). Bobin-Dubreux et al. (2) were the first to describe such an isolate from an outpatient who had not received glycopeptide antibiotics. To our knowledge, no staphylococci with reduced susceptibility or resistance to vancomycin have been isolated in the community or described so far.

The main objective of the present study is to report on the isolation of four VRS strains from healthy carriers inside and outside the hospital environment. We also studied these strains for the type of resistance to vancomycin and their patterns of resistance to other antibiotics and to investigate the mechanism of resistance to oxacillin.

MATERIALS AND METHODS

Bacterial strains and species identification. The four VRS strains described here were collected in an early surveillance study conducted in 2000 (unpublished data) among the workers of a private school ($n = 31$) and the staff of a general hospital ($n = 37$) with 127 beds, both located in Jaboticabal, Sao Paulo State, Brazil. The staphylococcal strains were obtained from saliva that had been collected with sterile swabs. The samples of saliva were cultivated in nutrient agar supplemented with 7.5% NaCl–1% egg emulsion. The plates were incubated at 35°C for 48 h. The staphylococcal strains were identified by colony morphology, Gram staining, and catalase testing. Five to six colonies from each plate with characteristic staphylococcal morphology were cultivated in 5% sheep blood agar and were identified by tube coagulase testing, by testing for DNase activity, and

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TABLE 1. Primers used in this study

Primer	Gene	Sequence (5′–3′)	Position[a]	Reference	GenBank accession no.
MecA1	*mecA*	CTCAGGTACTGCTATCCACC	4506–4525	1	Y14051
MecA2	*mecA*	CACTTGGTATATCTTCACC	4954–4936	1	Y14051
16S1	16S rRNA	CGAAAGCCTGACGGAGCAAC	378–397	15	AJ536434
16S2	16S rRNA	AACCTTGCGGTCGTACTCCC	520–501	15	AY144447
Coa1	*coa*	GAACAAAGCGGCCCCATCATTA	1301–1322	30	X17679
Coa2	*coa*	TAAGAAATATGCTCCGATTGTCG	2153–2131	30	X17679

[a] Positions in the gene sequence.

with an automated Microscan system (Dade Behring, West Sacramento, Calif.). The 164 original isolates were subcultured once onto 5% sheep blood agar, the vancomycin MICs for all isolates were determined by the E-test (AB Biodisk, Solna, Sweden) and broth dilution methods, and the isolates were stored at −70°C in skim milk.

The individuals who participated in this study were confirmed to be healthy by the physician responsible for hospital infection control at Santa Isabel Hospital. None of the individuals selected had received a glycopeptide or any other course of antimicrobial agent in the preceding 6 months.

Agar screening method for oxacillin susceptibility. Agar screening tests for susceptibility to oxacillin were performed as indicated by NCCLS guidelines (17). Quality control strains included *S. aureus* ATCC 29213 (negative control) and *S. aureus* ATCC 43300 (positive control).

Determination of MIC. The vancomycin MICs were determined by the E-test (AB Biodisk) with Mueller-Hinton agar and brain heart infusion (BHI) agar and by the broth dilution method with staphylococcal strains obtained from the initial isolation without consecutive cultures (named the parent strains) and with the revertants after 10 days of several passages in broth containing 4 μg of vancomycin per ml (named the derivative strains). The E-test with vancomycin was carried out in triplicate and according to the instructions of the manufacturer. The broth dilution method was undertaken precisely as described previously in NCCLS approved standard M7-A5 (17). Colonies were taken from overnight blood agar plates, and sterile Mueller-Hinton broth was inoculated to make a 0.5 McFarland suspension inoculum. The suspension inoculum was adjusted to 5×10^5 CFU/ml and was added to dilutions of vancomycin, the mixture was incubated at 35°C, and the cultures were read after 24 h of incubation.

The oxacillin MIC was determined by the E-test, performed according to the recommendations of the manufacturer (AB Biodisk). Mueller-Hinton agar plates containing 4% NaCl were inoculated by swabbing the surfaces with a 0.5× McFarland suspension for staphylococcal strains. E-test strips were placed on these plates, and the plates were then incubated at 35°C for 48 h (15).

The MICs of amikacin, erythromycin, ceftriaxone, cephalothin, trimethoprim-sulfamethoxazole, chloramphenicol, tetracycline, and quinopristin-dalfopristin were determined by the agar dilution method, as described by the NCCLS (17).

Control strains. *S. aureus* ATCC 29213 was used as a quality control strain for the agar dilution method. *S. aureus* strains ATCC 29213 and Mu 50 and *Enterococcus faecium* NCTC 7171 were used as quality control strains for determination of the vancomycin MICs by the E-test and broth dilution methods.

Detection of β-lactamase production. The staphylococcal strains were tested for β-lactamase production by the nitrocefin (Oxoid) test, according to the instructions of the manufacturer. *S. aureus* ATCC 29213 and *S. aureus* ATCC 25923 were used as positive and negative control strains for β-lactamase detection, respectively.

Amplification of *mecA*, *coa*, and 16S rRNA genes by PCR. The PCR method was carried out to detect the *mecA*, *coa*, and 16S rRNA genes. Chromosomal DNA of the strains was purified after lysis of the cells in the presence of lysozyme (100 mg/ml) and lysostaphin (1 mg/ml) by standard protocols (14). The primers used for amplification of the genes by PCR are reported in Table 1. PCR was carried out for 30 cycles of 60 s at 94°C, 30 s at 52°C, and 30 s at 72°C, with a final extension step at 72°C for 10 min. The same conditions were used for amplification of the 16S rRNA gene, except that the annealing temperature was 62°C. The amplified products were analyzed by 1.5% agarose gel electrophoresis, and the sizes of the amplification products were estimated by comparison with a 123-bp molecular size standard ladder. Strain *S. aureus* N315, a vancomycin-susceptible Japanese methicillin-resistant *S. aureus* MRSA strain supplied by Keiichi Hiramatsu (Juntendo University, Tokyo, Japan), was used as the control strain for the PCR amplification method.

Amplification of *van* genes by PCR. PCR detection of enterococcal *vanA*, *vanB*, and *vanC* genes was carried with DNA extracted from each strain as a template and with the primers described previously (9, 32). DNA amplifications were performed by using the following cycling parameters: denaturation at 94°C for 5 min, followed by 30 amplification cycles of 25 s at 94°C, 40 s at 52°C, and 50s at 72°C, with a final extension step at 72°C for 6 min. The resulting fragments were electrophoresed in a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light. Quality control strains included *E. faecium* NCTC 7171 (*vanA*), *Enterococcus faecalis* ATCC 51299 (*vanB*), *Enterococcus gallinarum* NCTC 12359 (*vanC1*), and *Enterococcus casseliflavus* NCTC 12361 (*vanC2*).

Southern blotting and hybridization. Southern blotting and hybridization were used to analyze the genomic DNA of isolates for the presence of the *mecA* and *van* genes. Cellular DNAs of VRS strains and control strains were extracted by the method described above. Cellular DNA (1 to 3 μg) of the staphylococcal strains was digested with HindIII, and SmaI was used for digestion of enterococcal DNA; this was followed by electrophoresis in a 1% agarose gel. DNA was transferred to nylon membranes and hybridized with a digoxigenin-labeled DNA probe specific for the *vanA*, *vanB*, *vanC*, and *mecA* genes. These probes were prepared by PCR from total cellular DNA of the enterococcal control strains cited above and *S. aureus* N315 with the primers and under the conditions mentioned above, except that the volume of dTTP (10 mM) was reduced and digoxigenin-labeled dUTP (1 mM) was included. Hybridization was performed according to the DIG system user's guide for filter hybridization (Boehringer, Mannheim, Germany).

Population analysis. Population analysis was performed for all four staphylococcal strains after they were subcultured in vancomycin-free medium for 10 days and also for derivative cells grown in the presence of 4 μg of vancomycin per ml at the same time. The term "derivative cells" has been applied to revertant cells subcultured in medium containing 4 μg of vancomycin per ml for 10 days. *S. aureus* ATCC 29213 was used as the negative control, and strains Mu50 and Mu3 were used as positive controls. The strains were cultured overnight in BHI broth. Then, an aliquot of the bacterial suspension was transferred to prewarmed BHI broth until a 0.5× McFarland standard was reached (10^8 CFU/ml); sevenfold dilutions of the cell suspensions were prepared with 0.9% sodium chloride. One hundred microliters of each dilution was used to inoculate BHI agar plates containing 0, 1, 2, 4, 6, 8, 12, 16, 24, 32, 64, 128, or 256 μg of vancomycin per ml. The plates were incubated at 37°C for 48 h, and the colonies were counted (13, 19).

PFGE. Clonal identity between the revertants and the isolates passaged in medium with 4 μg of vancomycin per ml was confirmed by pulsed-field gel electrophoresis (PFGE). Bacterial lysis, SmaI digestion of chromosomal DNA, and analysis of the DNA fragments by PFGE were carried out as described previously (11). Briefly, electrophoresis was performed in a Gene Navigator apparatus at 200 V for 25 h at 14°C. The equipment was adjusted for a pulse of 25 s for 20 h, 5 s for 4 h, and 0.5 s for 1 h. The banding patterns were visualized by ethidium bromide staining and UV transillumination. Isolate clonality was judged by visual comparison of the banding patterns of samples run together in the same gel by using previously described criteria (26).

Transmission electron microscopy. The procedures used for the preparation and examination of the staphylococcal cells by transmission electron microscopy were based on those published previously (7). However, some modifications were made for the preparation of the staphylococcal strains for analysis by transmission electron microscopy. The test bacterial strains were cultivated at 37°C for 18 h in BHI agar. The cells were further cultivated in BHI broth with shaking at 37°C for 4 h (control tubes). At the same time, the cells were cultivated in BHI broth containing 30 mM L-glutamine for 2 h at 37°C with shaking. After this time, 6 μg of vancomycin per ml was added to each tube except for the tube with

TABLE 2. MICs of several antimicrobials for VRS determined by the agar dilution, broth dilution, and E-test methods

Strains	MIC (μg/ml)[d]												
	VAN, broth dilution		E-test			Agar dilution method							
			VAN		OXA								
	P	D	P	D		CEF	CRO	CHL	SXT	AMK	ERY	TET	Q-D
S. haemolyticus 32S[a]	8	128	16	>256	≥256	≥256	≥256	≥256	≥256/4864	≥256	≥256	0.5	4
S. epidermidis 36S[a]	32	256	64	>256	≥256	128	128	8	4/76	64	≥256	32	0.5
S. capitis 62S[b]	4	16	8	16	0.125	≥256	4	≥256	4/76	4	≥256	0.125	0.5
S. capitis 67S[b]	16	32	32	32	0.125	≥256	1	8	0.5/9.5	2	≥256	0.5	1
S. aureus ATCC29213[c]	1	2	1	2	0.125	0.25	4	4	0.25/4.75	1	0.25	0.25	0.5

[a] Strains from the saliva of hospital staff.
[b] Strains from the saliva of school personell.
[c] Strain used for quality control.
[d] The broth dilution method and E-test with vancomycin were performed with the parent strains (P) and the derivative strains (D). The agar dilution method was performed according to the guidelines of the NCCLS (17). Antibiotic abbreviations: VAN, vancomycin; OXA, oxacillin; CEF, cephalothin; CRO, ceftriaxone; CHL, chloramphenicol; SXT, trimethoprim-sulfamethoxazole; AMK, amikacin; ERY, erythromycin; TET, tetracycline; Q-D, quinupristin-dalfopristin.

ATCC 29213, to which 1 μg of vancomycin per ml was added, and the tubes were further incubated at 37°C for 2 h with shaking. Morphometric evaluation of cell wall thickness was performed by using photographic images at a final magnification of ×9,700, and the cell wall thickness was measured as described previously (7). Thirty cells of each strain with nearly equatorial out surfaces were measured for the evaluation of cell wall thickness, and the results were expressed as the means ± standard deviations (SDs). The experiment was performed in duplicate on two independent occasions, and the results were presented as means ± SDs. Quality control strains included *S. aureus* ATCC 29213 and Mu50.

Statistical analysis of data. The statistical significance of the data was evaluated by Student's *t* test.

RESULTS

Identification of staphylococcal species. The staphylococcal strains characterized in the present study were identified as *S. capitis* subsp. *ureolyticus* 62S and *S. capitis* subsp. *ureolyticus* 67S, isolated from two different school staff members, and *S. haemolyticus* 32S and *S. epidermidis* 36S, isolated from two different hospital workers. Of the individuals studied, 98.5% were carriers of staphylococci and 76.5% were carriers of more than one species of staphylococci. Staphylococcal strains resistant to vancomycin represented 2.4% of the staphylococci tested.

Phenotypic resistance of staphylococcal strains. The MICs of the antimicrobial agents used, determined by the agar dilution, broth dilution, and the E-test methods, are summarized in Table 2. *S. haemolyticus* 32S was sensitive only to tetracycline, and *S. epidermidis* 36S was sensitive to chloramphenicol and quinupristin-dalfopristin. *S. capitis* 62S was sensitive to ceftriaxone, amikacin, oxacillin, tetracycline, and quinupristin-dalfopristin. *S. capitis* 67S was sensitive to ceftriaxone, chloramphenicol, trimethoprim-sulfamethoxazole, amikacin, oxacillin, quinupristin-dalfopristin, and tetracycline. Thus, the two isolates from hospital staff members were more resistant than the two isolates from school personnel. The vancomycin MICs determined with the E-test strips and by the broth dilution method showed that three of the staphylococcal strains were vancomycin resistant; the vancomycin MIC for *S. capitis* 62S was 4 μg/ml by broth dilution. The vancomycin MICs for the isolates were higher by the E-test with BHI agar than by the E-test with Mueller-Hinton agar (data not shown).

S. epidermidis 36S and *S. haemolyticus* 32S were oxacillin resistant, according to the oxacillin agar screening assay and the oxacillin MICs for the strains determined with the E-test strip. *S. capitis* strains 67S and 62S were not resistant to oxacillin (MICs < 0.5 μg/ml).

β-Lactamase production was detected in only two strains, *S. epidermidis* 36S and *S. haemolyticus* 32S (Table 3).

Genotypic resistance of staphylococcal strains. PCR was carried out with DNA extracted from staphylococcal strains and control strain *S. aureus* N315 as templates and with the set of 16S rRNA-specific primers. The expected band of 528 bp was amplified for all staphylococcal strains.

The *mecA* gene was detected in all isolates by the PCR amplification method. The positive result for the *mecA* gene for *S. capitis* 62S and *S. capitis* 67S showed discrepancies among conventional assays for the detection of methicillin resistance (Table 2). Southern blotting hybridization with a *mecA* gene-specific probe also detected the gene in all isolates (data not shown).

None of the staphylococcal isolates was found to carry the *vanA*, *vanB*, or *vanC* gene by PCR analysis (Table 3). Southern blotting hybridization with a probe specific for the *vanA*, *vanB*, and *vanC* genes also failed to detect the genes in the staphylococcal strains (data not shown).

Population analysis and PFGE of VRS strains. Figure 1 illustrates the results of the population analysis of the susceptible revertant isolates and the isolates passaged on medium containing vancomycin, which showed that the four derivative isolates were resistant to vancomycin, with 100% of the population growing in the presence of 4 μg of vancomycin per ml. The results of the analysis of SmaI restriction digestion of

TABLE 3. β-Lactamase production and PCR amplification of *mecA*, 16S rRNA, *coa*, and *van* genes of the VRS strains

Strains	β-Lactamase production by nitrocefin test	Gene amplification by PCR			
		mecA	*coa*	16S rRNA	*van*
S. haemolyticus 32S[a]	+	+	−	+	−
S. epidermidis 36S[a]	+	+	−	+	−
S. capitis 62S[b]	−	+	−	+	−
S. capitis 67S[b]	−	+	−	+	−

[a] Strains from the saliva of hospital staff.
[b] Strains from the saliva of school personnel.

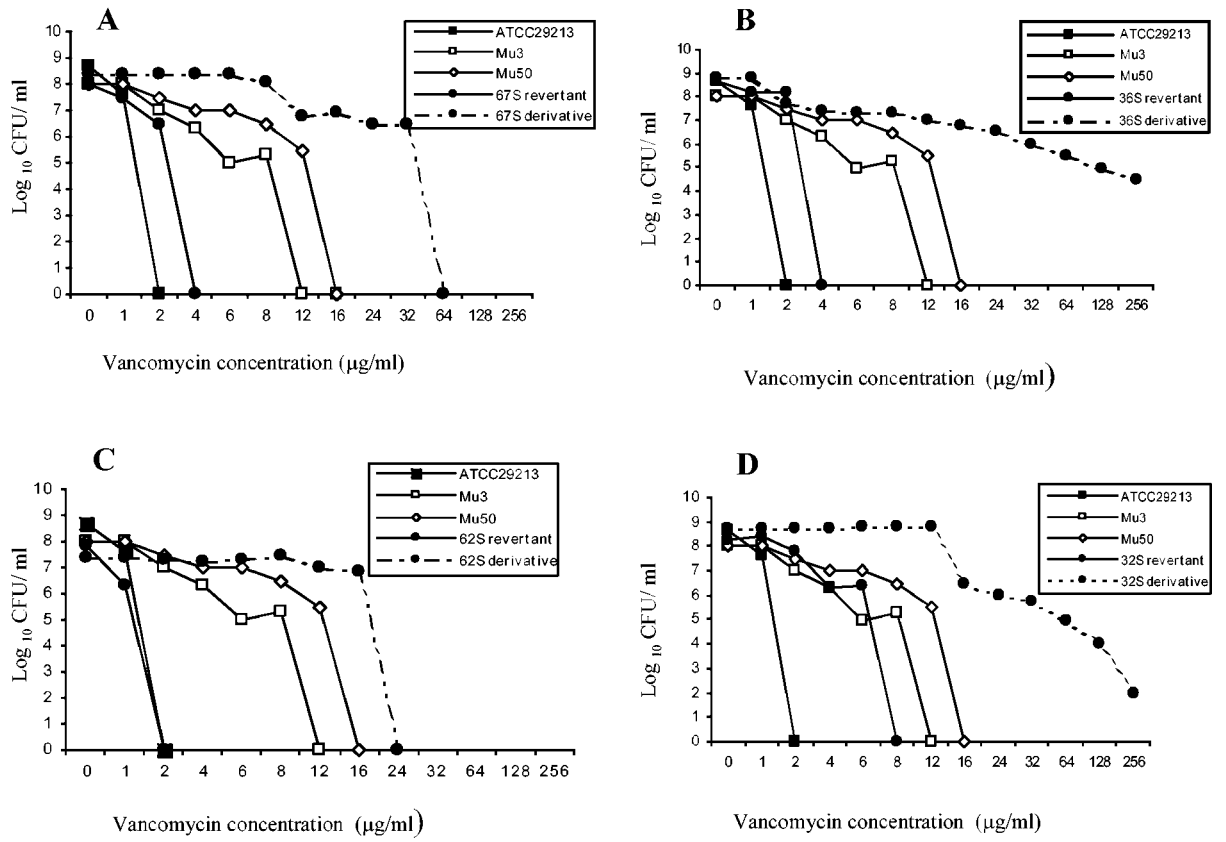

FIG. 1. Population analysis of the revertant strains and their derivatives. Strains Mu3, Mu50, and *S. aureus* ATCC 29213 were used as controls. (A) *S. capitis* 67S; (B) *S. epidermidis* 36S; (C) *S. capitis* 62S; (D) *S. haemolyticus* 32S. Strains grown overnight were serially diluted and plated on agar medium containing vancomycin at various concentrations. Derivative strains refer to the isolates obtained by daily subcultures of the revertants in medium containing vancomycin (4 μg/ml) for 10 days. Points appearing on the abscissa refer to 0 CFU.

genomic DNA by PFGE for the revertant strains and their derivative strains after consecutive passages in broth with vancomycin were indistinguishable (data not shown).

The vancomycin-resistant staphylococcal strains returned to susceptible levels (vancomycin MICs ≤ 2 μg/ml) after 10 days of serial passage in a drug-free medium. However, VRS strains were selected at very high frequencies (10^{-2} and 10^{-3}) when the revertants were exposed to 4 μg of vancomycin/ml. *S. aureus* ATCC 29213, the susceptible quality control strain, was also subjected to population analysis after exposure to vancomycin. The vancomycin MIC for the strain increased after exposure to vancomycin but did not survive in the presence of more than 2 μg of vancomycin/ml.

Cell wall thickness and vancomycin resistance. All vancomycin-resistant staphylococcal strains and two control strains (one vancomycin-susceptible *S. aureus* [VSSA] strain, ATCC 29213, and one VRSA strain, Mu50) were subjected to a morphometric study by transmission electron microscopy. Figure 2 shows transmission electron micrographs of the staphylococcal strains grown in BHI medium and in BHI medium with vancomycin. As is evident in Fig. 2, the staphylococcal strains studied and strain Mu50 had significantly thicker cell walls than the VSSA strain used as a control. The differences in the cell wall thickness between Mu50 and the vancomycin-resistant staphylococcal strains (strains 32S, 36S, 62S, and 67S) and the VSSA strain were all statistically significant ($P < 0.001$ for all combinations). As shown in Fig. 2, the cell walls of the staphylococcal strains studied became thickest when vancomycin was added to BHI medium ($P < 0.001$ for all comparisons), while no thickening effect was observed in strain ATCC 29213 by the addition of vancomycin to BHI medium ($P = 0.279$).

DISCUSSION

Despite the extensive literature about the carriage of *S. aureus* and CoNS in the nose and on the hands, relatively little attention has been paid to the oral cavity as a reservoir for these organisms. The species of staphylococci most frequently reported from oral samples are *S. epidermidis* and *S. aureus*; but *S. capitis*, *S. saprophyticus*, *S. warneri*, *S. haemolyticus*, *S. xylosus*, and *S. simulans* have also been reported from oral samples (25). Disseminated oral staphylococcal strains have the potential to recolonize other body sites or to be a source of cross-infection for other individuals. During a surveillance study of carriers of staphylococci inside and outside the hospital environment, four vancomycin-resistant staphylococcal strains were isolated from saliva and studied in detail.

The majority of staphylococci heterogeneously resistant to vancomycin isolated so far were methicillin and oxacillin resistant, showing that resistance to vancomycin is associated with

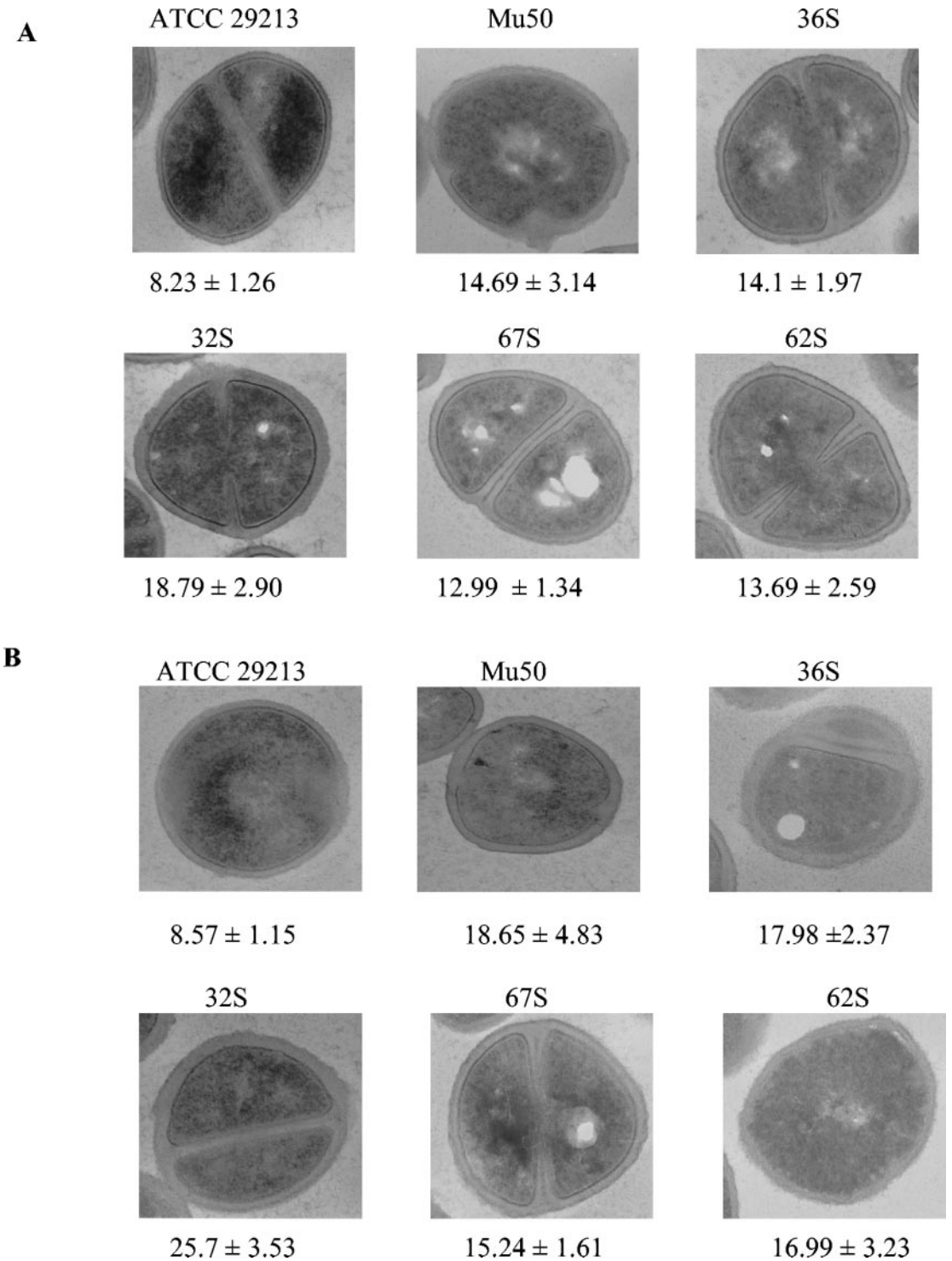

FIG. 2. Comparison of cell wall thicknesses among the test strains after cultivation in BHI medium and BHI medium supplemented with L-glutamine and vancomycin. (A and B) Comparison of cell-wall thicknesses of the staphylococcal strains cultivated in BHI medium (A) and after incubation in BHI medium supplemented with 30 mM L-glutamine and vancomycin (6 μg/ml for all strains except strain ATCC 29213, to which 1 μg of vancomycin/ml was added). The values under each panel are mean ± SD thicknesses (in nanometers). The bacterial cultures in BHI medium were divided into two portions. One portion was cultivated in BHI broth at 37°C for 4 h and subjected to electron microscopy (A). The other portion was cultivated in BHI broth supplemented with 30 mM L-glutamine at 37°C for 2 h, vancomycin was added to the final concentration mentioned above, and the cells were incubated for 2 h and then subjected to electron microscopic examination (B).

resistance to oxacillin (12). The vancomycin-resistant staphylococcal strains obtained in this study showed different patterns of susceptibility to oxacillin. The recent isolation of a vancomycin-resistant, oxacillin-susceptible strain further indicated that vancomycin resistance is not necessarily confined to MRSA and that the mechanism of resistance in vancomycin-intermediate *S. aureus* strains may involve alterations in cell wall metabolism, which thus affects the activities of β-lactam antibiotics (2, 24). A study examining the phenotypic characteristics of a laboratory-generated mutant of an MRSA strain with high-level vancomycin resistance revealed that the vancomycin resistance had been achieved at the expense of β-lactam resistance (23). According to Hiramatsu (12), the precise deletion of the SCC *mec* element carrying the *mecA* gene from strains Mu50 and Mu3 and other Japanese hetero-VRSA strains did not alter the levels or patterns of vancomycin resistance.

According to the results of the population analysis, all the staphylococcal strains showed heteroresistance to vancomycin, and this resistance proved to be unstable. Even though *S. haemolyticus* strain 32S had apparently shown more heteroresistance than the *S. capitis* strains (strains 62S and 67S), it was able to grow in the presence of a higher concentration of vancomycin. The reversal of glycopeptide resistance, which returned to susceptible levels, occurred with all VRS isolates after 10 days of serial passage on nonselective medium. Boyle-Vavra et al. (3) reported this phenomenon and, considering the tendency of strains to revert, stated that glycopeptide-intermediate *S. aureus* isolates should be maintained in vancomycin-containing medium to prevent reversion. Exposure of these revertants to 4 μg of vancomycin/ml selected for VRS strains, named derivative strains, at very high frequencies after 10 days of serial passage. This finding is very important, because although these vancomycin-resistant staphylococcal strains may not disseminate with stable resistance, they can readily revert to vancomycin resistance when they are exposed to vancomycin.

The mechanism of resistance to vancomycin in these staphylococcal strains is not *van* gene mediated, because the *vanA*, *vanB*, and *vanC* genes were not found. Although the precise genetic mechanism for vancomycin resistance in these staphylococcal strains awaits elucidation, the thickening of the cell wall may have contributed to the vancomycin resistance in the staphylococcal strains studied. The thickening effect in the cell wall of the vancomycin-resistant staphylococcal strains studied and in strain Mu50 was observed by the addition of vancomycin to BHI broth containing 30 mM L-glutamine. The supplementation of the culture medium with glutamine should further enhance the utilization of glucose as a cell wall precursor metabolite after vancomycin addition to BHI broth. We think that the thickening effect could be enhanced by using a medium containing the amino acids essential for cell growth and cell wall synthesis and a high concentration of glucose, as observed by Cui et al. (7). No thickening effect was observed for the cell wall of the VSSA strain after vancomycin addition to BHI broth. We cannot exclude the possibility that other mechanisms or metabolic alterations occurred in these strains because the cell wall thickening could not be the only cause of the high vancomycin MICs obtained after daily subculture under selective pressure with this drug. According to Schaaff et al. (22), an elevated mutation frequency could be one of the factors in the chromosomal background of *S. aureus* that favors the emergence of vancomycin resistance.

Vancomycin-intermediate *S. aureus* isolates were first found in nature more than 15 years ago while investigators were screening isolates for vancomycin susceptibility (31). Added to this, several hetero-VRSA strains were found in the late 1980s in Japan before the introduction of vancomycin (12). We suppose that the expression of vancomycin resistance in staphylococci is due to several metabolic alterations, and the presence of alterations in the chromosomal backgrounds of some strains could favor the development of vancomycin resistance. This genetic background would have been acquired over time. Antimicrobial agents or other substances with similar functions could certainly select those bacteria that have this genetic background and that are frequently multiresistant. However, exposure to glycopeptide antibiotics would certainly appear to play an important role.

In conclusion, vancomycin resistance expression in the CoNS isolates studied proved to be unstable, with strains tending to return to a vancomycin resistance status when they were exposed to vancomycin. The resistance to vancomycin in CoNS is not restricted to oxacillin-resistant staphylococcal strains. The mechanism of resistance to vancomycin in CoNS strains awaits elucidation; however, the thickness of the cell wall is one of the factors that favors the emergence of vancomycin resistance. The presence of these VRS strains in carriers is a source of concern, especially when the fact that these multiresistant strains are not restricted to the hospital environment is considered.

ACKNOWLEDGMENTS

We especially thank Neil Woodford (Central Public Health Laboratory Service, Colindale, London, United Kingdom) for supplying the enterococcal strains and Keiichi Hiramatsu (Juntendo University) for supplying *S. aureus* strains N315, Mu50, and Mu3 and confirming the vancomycin MICs for the staphylococcal strains researched in this study. We are grateful to Luis Eduardo Gerbasi, President of the Hospital Infection Control Commission of Hospital Santa Isabel, and also to Joseane Cristina Ferreira for technical assistance during the PFGE assay. We are grateful to technicians (Maria Dolores S. Pereira and José Augusto Maulin) of the Departament of Biologia Celular and Molecular and Bioagentes Patogênicos of the Faculty of Medicine of Ribeirão Preto (USP) for performing electron microscopy of the staphylococcal strains.

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