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EXPERIMENTAL ARTICLES

Physiological Properties of the Vancomycin-Resistant Strain *Staphylococcus epidermidis* 33 GISK VAN^R

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Abstract—Physiological properties of a selected *Staphylococcus epidermidis* strain 33 GISK VAN^R with high resistance to vancomycin and multiple resistance to various antibiotics, as well as decreased sensitivity to lysozyme, lysostaphin, and the low-molecular mass peptide warnerin were studied. The strain was characterized by a thicker cell wall, resulting in considerably decreased rates of vancomycin penetration into the cells and in limited access of the antibiotic to its targets on the bacterial membrane.

Keywords: coagulase-negative staphylococci, vancomycin, warnerin

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Coagulase-negative staphylococci (CNS) are a part of habitual human and animal microbiota; they may cause diseases in the case of imbalance of the host immune system caused by viral infections, contamination of implanted medical devices, or inadequate antibacterial treatment. Staphylococci are known to possess high capability for adaptation to antibiotics, e.g., to the glycopeptide antibiotic vancomycin in the case of its irrational application against gram-positive microflora [1]. A rapid increase in the number of staphylococcal strains characterized by heteroresistance to this antibiotic was observed in many countries [2, 3]. At present, bacteria are considered to be sensitive, middle-resistant, or resistant to vancomycin when its minimal inhibitory concentration (MIC) are ≤2, 4–8, or ≥16 μg/mL, respectively [4]. The basic genetic mechanism responsible for resistance of staphylococci to vancomycin remains unclear; it may be associated with thickening of bacterial cell walls [5–7] and with increased number of free D-alanyl-D-alanine residues in the peptidoglycan molecule. These events may promote considerable nonspecific binding of this antibiotic, thus limiting its access to real targets on the cytoplasmic membrane and, therefore, may evoke increased bacterial resistance to vancomycin [8]. Importantly, clinical strains of *S. aureus* and CNS with decreased sensitivity to vancomycin were revealed in the patients treated with both glycopeptides and other antibiotics [9–13]. At the same time, strains of staphylococci heteroresistant to vancomycin have been isolated long before practical application of this antibiotic [14]. Emergence of vancomycin resistance in staphylococci seems to be due to some metabolic reconstructions, and genetic abnormalities in these strains can be accumulated for a long time [15]. There is a hypothesis that changes in the physiology of staphylococci due to accumulation of mutations may result in the emergence of a vancomycin-resistant phenotype [16]. Indeed, genetic analysis of a number of vancomycin-resistant strains revealed changes in expression of many genes, in particular, the *pbp* genes encoding penicillin-binding proteins involved in the cell wall formation [17], the *mut* genes responsible for the mutation frequency [18], the *mprF* gene encoding lysylphosphatidylglycerol synthase [19], a group of *gra* genes associated with resistance to glycopeptides [20] and encoding the two-component GraRS system (which controls the operation of a number of genes including those involved in the cell wall synthesis) [21], a group of the *vra* genes of the two-component sensory system VraRS (which is involved in the regulation of peptidoglycan synthesis) [22], the *agr* locus (accessory gene regulator) responsible for operation of the quorum-sensing system [23], the *wal* regulon, which regulates cell division [24], and other key genes [20, 25, 26].

The goal of the present work was to investigate the changes in the physiological properties of *S. epidermidis* strain 33 GISK in the course of acquisition of vancomycin resistance, to elucidate the mechanisms responsible for this process, and to develop the methods for inhibition of the strains resistant to this antibiotic.

MATERIALS AND METHODS

The study was carried out with the strain *S. epidermidis* 33 GISK deposited in the State Collection of Pathogenic Microorganisms (SCPM), Scientific Center for Expertise of Means of Medical Application,

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Ministry of Health of the Russian Federation and with the vancomycin-resistant variant *S. epidermidis* 33 GISK Van^r, which was selected by cultivation of the parent strain in liquid LB media with increasing concentrations of antibiotic beginning from 0.5 μg/mL (MIC of vancomycin for this strain) [27].

At each stage of selection, bacteria were once cultivated on LB agar medium with an appropriate concentration of the antibiotic to establish the antibiotic resistance by means of colony growth.

Bacteria were cultivated on a Certomat IS orbital shaker (Sartorius, Germany) (150 rpm) at 37°C. Cell growth was monitored by measuring optical density of the culture aliquots at 600 nm on a PD-303 spectrophotometer (APEL, Japan).

The minimal inhibitory concentrations of antibacterial preparations—vancomycin (Sigma, United States), cefazolin (Biosintez, Russia), linezolid (Fresenius Kabi Norge AS, Norway), daptomycin (Novartis Pharma AG, Switzerland), clarithromycin (Abbott France, France), chloramphenicol (Sigma, United States), ciprofloxacin (Promed Exports, India), gentamycin (KRKA, Slovenia), rifampicin (Ferein, Russia), bacitracin (Sigma, United States), polymyxin B (Sigma, United States), colistin (Sigma, United States), lysozyme (Sigma, United States), lysostaphin (Sigma, United States)—and the low-molecular-weight cationic peptide warnerin for the studied strains were determined using twofold serial dilutions in 96-well polystyrene immunological plates (Medpolimer, Russia). The exponential-phase cells were inoculated into the wells with LB medium to the final concentration of 5×10^5 CFU/mL. The sensitivity to antibiotics was assayed the by the disk diffusion method (NITsF, Russia) [4].

Retention of vancomycin resistance in the obtained strain and its sensitivity to other antibacterial preparations were assayed after 20 successive passages of the culture in liquid LB medium by using the method of twofold serial dilutions and the disc-diffusion technique [4].

To perform the population analysis of bacterial cultures, the modified method described in [28] was used: aliquots of tenfold dilutions of bacterial suspensions were applied onto agar media with different concentrations of vancomycin [29]. The cultures of both vancomycin-sensitive and vancomycin-resistant strains grown in liquid LB medium for 18 h were diluted with the medium to the concentration of 10^8 CFU/mL, and tenfold dilutions of these suspensions were spread over the plates with LB agar containing vancomycin in concentrations of 0, 1, 2, and 4 μg/mL (for *S. epidermidis* 33 GISK) or 0, 4, 8, 16, 32, 64, 128, 256, and 512 μg/mL (for *S. epidermidis* 33 GISK Van^r). The plates were incubated at 37°C for 4 days; the number of resistant cells was calculated from the number of colonies grown.

To determine the time course of vancomycin binding to bacterial cells, vancomycin (65 μg/mL) was added to the exponential-phase cultures of sensitive and resistant strains, and cultivation was continued for 72 h under the above-mentioned conditions. To determine vancomycin concentration, an aliquot of the culture liquid was centrifuged (16000 *g*, 10 min), the supernatant was sterilized by filtration through 0.45-μm membrane filters (Millipore, United States), and vancomycin concentration was determined by the method of twofold dilutions in microplates with *S. epidermidis* 33 GISK as the test culture. Simultaneously, vancomycin concentration in the growth medium was determined by measuring optical density of the supernatants at 240 nm by using an Áktapurifier system (GE Healthcare, Sweden) equipped with a μRPC C2/C18 ST 4.6/100 column under a 0–70% concentration gradient of acetonitrile (Kriokchrom, Russia) in 0.1% trifluoroacetic acid (Fluka, United States) at an elution rate of 1.2 mL/min.

The intensity of the gentian violet binding by bacterial cells was determined by addition of 0.01% solution of gentian violet in 10 mM phosphate buffer (pH 7.2) to the cell suspensions; after a 20-min incubation at room temperature, bacterial cells were precipitated by centrifugation (16000 *g*, 10 min), washed twice with 0.14 M NaCl, and the pellet was extracted with ethanol; absorption of the ethanol extract was measured at 570 nm on a PD-303 spectrophotometer (APEL, Japan).

Morphology of bacterial cells was examined by atomic force microscopy. The cells of an exponential-phase culture grown in LB medium were precipitated by centrifugation (3500 *g*, 5 min), washed twice with 10 mM phosphate buffer (pH 7.2), and fixed with 2.5% solution of glutaraldehyde in the same buffer for 1 h; after washing, the cells were resuspended in the same buffer, placed onto an object slide, dried at room temperature, and examined under an AFM Nano-DST microscope (Pacific nanotechnology, United States).

The size of Gram-stained cells was measured in several fields of view under a Mikrovizor μVizo-103 microscope (LOMO, Russia); the cell shape was accepted as spherical, and approximate surface area of the cells was calculated accordingly.

RESULTS

The vancomycin-resistant strain *S. epidermidis* 33 GISK Van^r was obtained after 16 successive transfers of strain *S. epidermidis* 33 GISK in liquid LB medium with concentrations of vancomycin (up to 32 μg/mL); the antibiotic concentration was increased stepwise with intervals from 0.5 to 3.0, mainly 1.0–2.0 μg/mL, depending on the growth intensity at the previous antibiotic concentration (the first stage of selection).

At this stage, diameters of the growth inhibition zones formed around the discs containing 30 μg of vancomycin were equal for both the isolate and the parent strain. In spite of enrichment of the population with more resistant cells in the course of selection, the disc diffusion method was unsuitable for detection of a small decrease in the cell sensitivity to vancomycin [25]. Therefore, the results obtained by this method should be considered approximate, and other methods for evaluation of the vancomycin resistance should be applied. Nevertheless, the obtained isolate could be considered a vancomycin-resistant strain since it was able to grow in the presence of a relatively high concentration of vancomycin (32 μg/mL). However, in the second stage of selection, when this isolate was cultivated at this antibiotic concentration both in liquid and on solid media during ten transfers, the growth suppression zones formed around discs were gradually decreased and finally disappeared. The time course of the strain growth at this stage showed a decrease in the biomass accumulation as well as an increase in the generation time (88 ± 5 min) compared to that of the parent strain (73 ± 5 min). The data on biomass level and the number of viable cells of both the parent and resistant strains in the course of their growth in LB medium are shown on Fig. 1.

The atomic force microscopic examination revealed considerable changes in the cell morphology of the resistant strain (an increase in the cell volume and a change in the relief of the outer cell surface) as compared with those of the parent strain (Fig. 2).

According to the quantitative population analysis, bacteria of the parent strain *S. epidermidis* 33 GISK were rather homogeneous in their sensitivity to vancomycin and contained no subpopulations with the MIC of vancomycin exceeding >4 μg/mL (Fig. 3). At the same time, strain Van^r was characterized by pronounced heterogeneity in vancomycin resistance; bacteria capable of forming colonies on agar media with 64, 128, and 256 μg/mL of vancomycin were revealed at the frequency of 10^{-3}, 10^{-4}, and 10^{-6}, respectively.

The level of vancomycin resistance in bacteria was assayed both by the disc diffusion method and by determination of MIC values in the serial dilutions of the cell suspension. The parent strain *S. epidermidis* 33 GISK was found to be sensitive to vancomycin, since the growth suppression zones formed around the discs with this antibiotic had diameters ≥15 mm, corresponding to MIC of ≤4 μg/mL [4]. The value of MIC determined by the titration method ranged from 0.49 to 0.98 μg/mL.

The selected strain was resistant to vancomycin; it was evident from the absence of growth inhibition zones around the discs with antibiotic that corresponded to MIC of ≥32 μg/mL. The MIC value determined by the titration method was 500 μg/mL; growth was observed in the wells containing up to 250 μg/mL of vancomycin; therefore, a true value of MIC appeared to be within the range of vancomycin concentrations from 250 to 500 μg/mL. Thus, the level of vancomycin resistance of strain *S. epidermidis* 33GISK Van^r revealed in our experiments was the maximum value characteristic of the minor portion of the population. Importantly, no marked changes in the acquired level of the vancomycin resistance were observed in strain *S. epidermidis* 33GISK Van^r when bacteria were grown during 20 successive transfers in antibiotic-free medium.

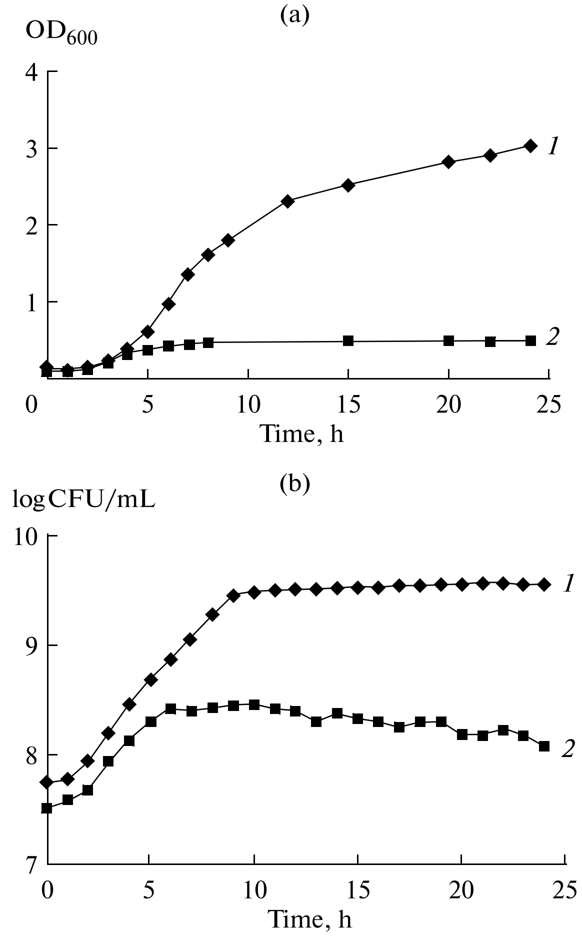

Fig. 1. Growth characteristics of *S. epidermidis* 33 GISK (*1*) and *S. epidermidis* 33 GISK Van^r (*2*): changes in optical densities of the cultures (a) and the time course of the number of viable cells (CFU) (b).

In special experiments, it was found that the vancomycin-resistant strain *S. epidermidis* 33GISK Van^r isolated at the first stage differed considerably from the parent strain in its sensitivity to various bacteriolytic factors: similarly to the parent strain, it was sensitive to lysostaphin, but was characterized by a 4-fold higher resistance to lysozyme. After several transfers in liquid and on agar media with 32 μg/mL of vancomycin (the second stage of isolation), the isolate acquired resistance to lysostaphin (MIC value increased by more than 500 times) and even more pronounced resistance to lysozyme (Table 1).

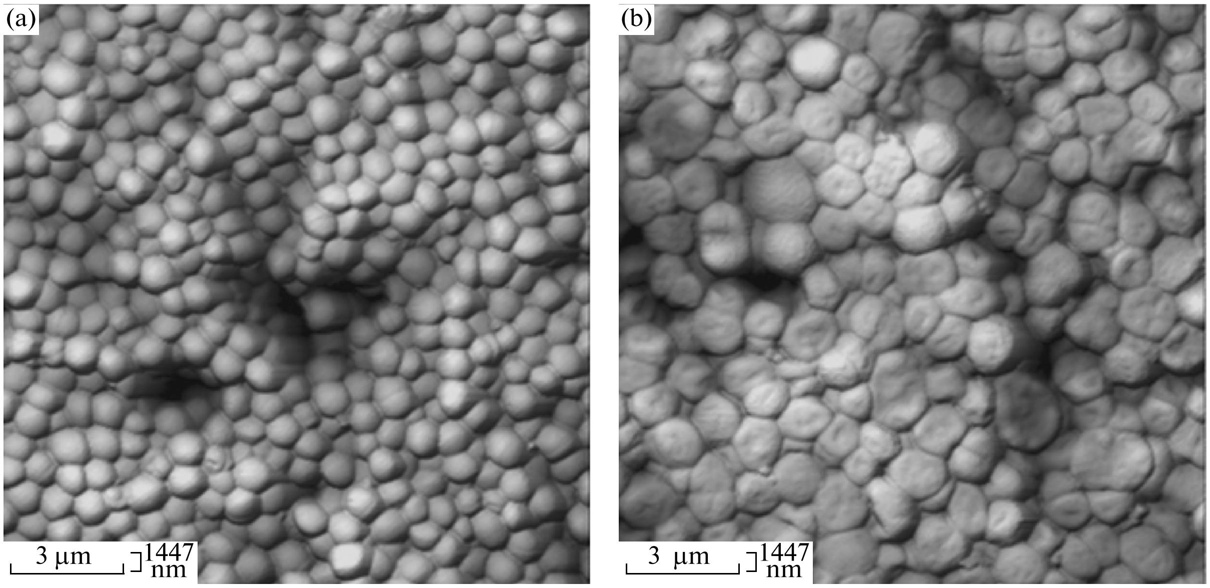

Fig. 2. Atomic force microscopy of the cells: *S. epidermidis* 33 GISK (a) and *S. epidermidis* 33 GISK Van^r (b).

It is important that the isolate became also resistant to the low-molecular-weight cationic peptide warnerin; however, although MIC of this peptide was 32 times higher than that for the parent strain, the level of the isolate resistance to warnerin remained an order of magnitude lower than that to lysozyme and lysostaphin (Table 1).

Moreover, strain Van^r simultaneously acquired resistance to the beta-lactam antibiotics (benzylpenicillin, cefazolin, and oxacillin), lincomycin, linezolid, daptomycin, fusidin, and gentamycin, but became more sensitive to macrolides (erythromycin and clarithromycin), chloramphenicol, and peptide antibiotics (bacitracin, polymyxin B, and colistin). It should be noted that the sensitivity of the isolate to tetracycline and rifampicin remained at the same level as that of the parent strain (Table 2).

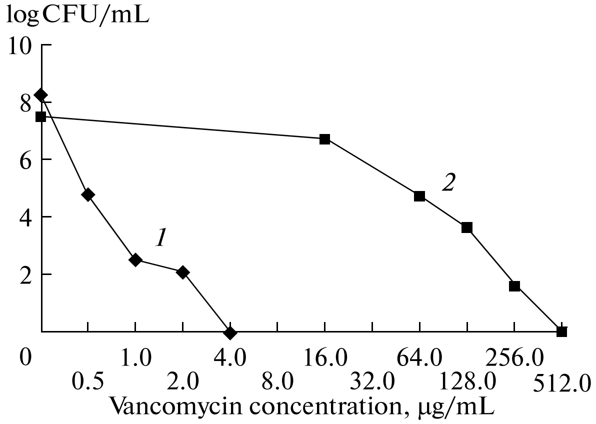

Fig. 3. Population analysis of vancomycin sensitivity of strains *S. epidermidis* 33 GISK (*1*) and *S. epidermidis* 33 GISK Van^r (*2*).

The revealed changes in the sensitivity of strain Van^r to various antibiotics were probably associated with the physicochemical characteristics of bacterial cell walls. As seen from Fig. 4, the cells of strain *S. epidermidis* 33 GISK adsorbed a considerably lower amount of gentian violet than strain Van^r, which may be due to the presence of more pronounced cell walls.

Based on a dependence of optical density of alcohol solutions of gentian violet on their concentrations, we calculated an approximate dye amount, which was bound by one bacterial cell of each strain. It was found that a cell of *S. epidermidis* 33 GISK adsorbed 1.7 × 10^7 molecules of gentian violet, whereas a cell of *S. epidermidis* 33 GISK Van^r adsorbed an order of magnitude larger number of the dye molecules (~1.4 × 10^8), which can possibly be due to a larger average size of the Van^r cells. However, the calculated surface area of one cell of the resistant strain (~7 µm^2) was only four times larger than that of the parent strain (~1.8 µm^2). Thus, the Van^r cells appeared to adsorb a higher dye amount because of not only increased cell size, but also due to the thickening of the cell wall and to an increase in its mass.

The data on vancomycin sorption by strains *S. epidermidis* 33 GISK and *S. epidermidis* 33 GISK Van^r are shown on Figs. 5 and 6. In both cases, 65 µg/mL of vancomycin was added to the exponential-phase-cultures. As can be seen from Fig. 1, the studied exponential-phase cultures differed in both optical density and the amount of viable cells. Addition of vancomycin had different effects on biomass accumulation by two cultures (Fig. 6). During the first two hours after addition of the antibiotic, the number of cells increased by 1.5-fold in the parent strain (Fig. 5a) and by 15 times in Van^r strain (Fig. 5b), which indicated a decrease in the inhibitory effect of vancomycin on the cell division in the resistant strain. Two hours after addition of the antibiotic to the parent strain, its concentration in medium decreased to 45 μg/mL and remained at this level for 72 h (Fig. 5a). A possible explanation of this fact is that the cell walls of the sensitive strain were rapidly saturated by vancomycin and then the antibiotic adsorption was stopped. In the case of the resistant strain, vancomycin concentration in the medium did not decrease that sharply, reaching ~50 μg/mL at the same period of cultivation (Fig. 5b).

DISCUSSION

Bacteria of the genus *Staphylococcus* are among the most dangerous human and animal pathogens; therefore, the study of conditions responsible for development of their resistance to various antibiotic agents is of great importance. Although bacteria belonging to the group of coagulase-negative staphylococci are less virulent than *S. aureus*, they are responsible for an increasing number of nosocomial infections, especially when invasive devices are used. This dictates the necessity of detailed study of the biological features of CNS which may be associated with development of bacterial resistance to glycopeptide antibiotics. Vancomycin, a member of this group, is used for suppression of acute infections caused by methicillin-resistant staphylococci and other gram-positive microorganisms. Unfortunately, wide application of vancomycin results in the selection of bacterial strains resistant to this

Table 1. Sensitivity of *S. epidermidis* 33 GISK and *S. epidermidis* 33 GISK Van^r to bacteriolytic factors

Bacteriolytic factors	MIC, μg/mL	
	S. epidermidis 33 GISK	*S. epidermidis* 33 GISK Van^r
Warnerin	0.25	≥8
Lysozyme	1	≥250
Lysostaphin	≤0.25	≥125

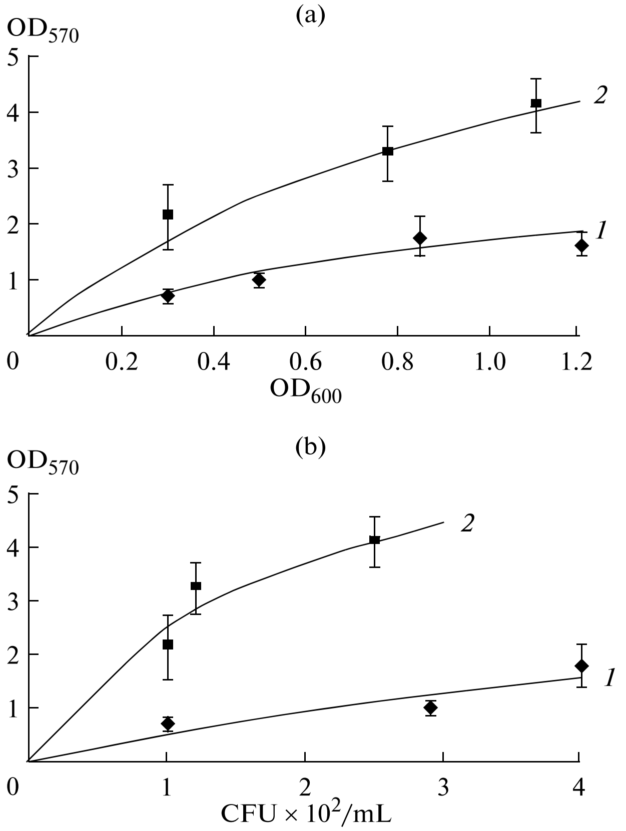

Fig. 4. Adsorption of gentian violet by the cell suspensions of *S. epidermidis* 33 GISK (*1*) and *S. epidermidis* 33 GISK Van^r (*2*) depending on cell density (a) and the number of viable cells (b).

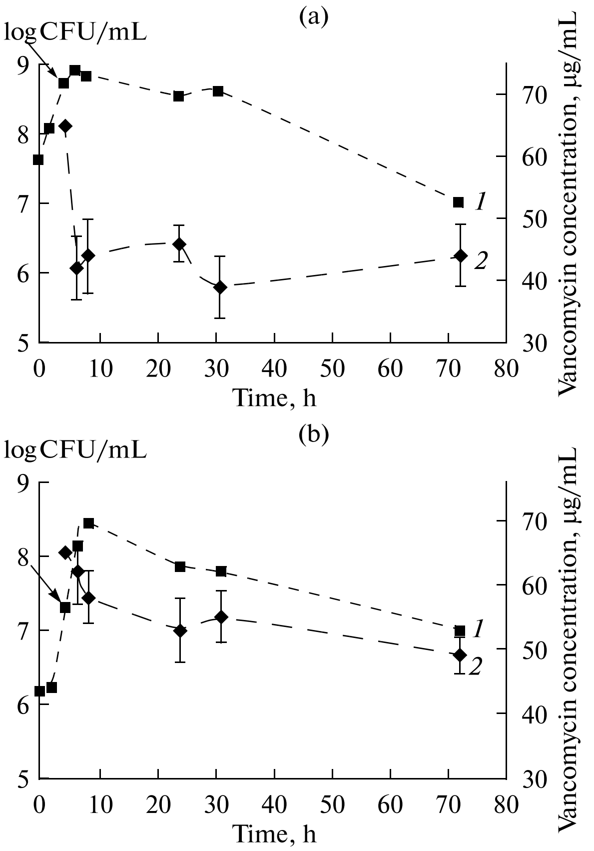

Fig. 5. Vancomycin adsorption in the course of cultivation of *S. epidermidis* 33 GISK (a) and *S. epidermidis* 33 GISK Van^r (b): CFU value during cell incubation with vancomycin (*1*) and vancomycin concentration in the medium (*2*). Arrows indicate the time of vancomycin addition.

Table 2. Sensitivity of *S. epidermidis* 33 GISK and *S. epidermidis* 33 GISK Van^r to antibiotics

Antibiotics	MIC, μg/mL	
	S. epidermidis 33 GISK	*S. epidermidis* 33 GISKVan^r
	Increased level of antibiotic resistance	
Glycopeptides		
Vancomycin	0.49–0.98	125–250
Beta-lactams		
Benzylpenicillin	≤0.12*	≥0.25*
Cefazolin	1–2	31.25–62.5
Oxacillin	≤0.25	≥0.5*
Lincosamides		
Lincomycin	≤2*	≥8*
Oxazolidinones		
Linezolid	1.96	7.8
Lipopeptides		
Daptomycin	≤1	350–700
Fusidin	≤0.5*	≥2*
Aminoglycosides		
Gentamycin	≤0.002	0.1–0.2
	Increased level of the antibiotic sensitivity	
Macrolides		
Erythromycin	≤0.5*	<0.5*
Clarithromycin	1.96	0.06–0.12
Phenicols		
Chloramphenicol	7.8–15.6	0.98–1.96
Polypeptides		
Bacitracin	7.8–31.25	0.49–0.98
Polymyxin B	0.98–1.96	0.00006–0.0001
Colistin	0.98–3.9	0.00006–0.0001
	Retained level of antibiotic sensitivity	
Tetracycline	≤4*	≤4*
Rifampicin	≤1*	≤1*

* Results of the disc-diffusion test.

antibiotic. It is important that mechanisms responsible for vancomycin resistance of CNS are close to those operating in the *S. aureus* strains [8, 30].

Comparative studies of the physiological properties of *S. epidermidis* strain 33 GISK and its vancomycin-resistant variant *S. epidermidis* 33 GISK Van^r revealed that the cells of the latter strain were heterogeneous in their sensitivity to this antibiotic.

It was found that the cells of *S. epidermidis* 33 GISK Van^r were also more heterogeneous in their size than those of the parent strain. Their shape was different and the surface was nonhomogenous. Examination of stained specimens of these bacteria revealed the cells agglomerated in tetrads, which was atypical of staphylococci; this formation could result from impairment of the cell division regulation, possibly because of a change in the functional activity of peptidoglycan hydrolases [31–33]. Formation of the Van^r phenotype also resulted in a change in the culture sensitivity to antibacterial preparations affecting the synthesis of the cell wall components and proteins.

There are only scanty literature data on antibiotic resistance of the clinical isolates of staphylococci with decreased sensitivity to glycopeptide antibiotics. A change in the sensitivity of vancomycin-resistant staphylococci to antibacterial preparations cannot be explained by an increase in the cell size and thickening of bacterial cell walls alone. As a rule, vancomycin-resistant bacteria exhibited methicillin resistance [3, 7, 9, 14, 15], and some of them were capable of overproduction of penicillin-binding proteins; others showed decreased affinity to beta-lactam antibiotics [17].

A decrease in the sensitivity of vancomycin-resistant bacterial strains to a lipopeptide antibiotic daptomycin could be due to the cell wall thickening that formed a physical barrier for penetration of large molecules of the antibiotic to cytoplasmic membrane; it was also probably associated with a decrease in the total negative charge of bacterial cells at the expense of increased content of lysylphosphatidylglycerol in the membranes that possibly resulted in weakened interaction with positively charged daptomycin molecules in a complex with Ca^{2+} ions [34].

Although diameters of the growth inhibition zones formed around gentamycin-containing discs were similar for both the parent and the vancomycin-resistant strains (MIC value of ≤4 μg/mL), determination of gentamycin sensitivity by the method of serial dilutions showed the MIC value for the isolate to be 0.1–0.2 μg/mL, which was two orders of magnitude higher than that for the parent strain (0.002 μg/mL). It can be possibly due to decreased attraction of the cationic molecules of gentamycin to the Van^r cells because of an increased level of lysylphosphatidylglycerol in their membranes [34]. This mechanism can explain increased MIC of a polycationic peptide warnerin for vancomycin-resistant strain.

A decrease in MIC of some antibiotics during the development of vancomycin resistance appeared to be associated with certain changes in bacterial metabolism. At the same time, the cell wall thickening showed no effect on penetration of macrolides (erythromycin and clarithromycin), peptide antibiotics (bacitracin and polymyxins), and chloramphenicol into bacterial cells. The mechanism of action of macrolides is similar to that of lincosamides; it is known that resistance to these antibiotics is widespread among methicillin-resistant staphylococci [35]; however, in our experiments, development of only lincomycin resistance was established.

A decrease in the rate of vancomycin sorption by the Van^r cells was possibly due to retarded saturation of the cell wall because of its thickening, which was revealed in experiments on gentian violet adsorption by the vancomycin-resistant strain, and, as a result, by decreased access of the antibiotic to its membrane targets.

Thus, the results of this study indicate that development of resistance to a glycopeptide antibiotic vancomycin in bacteria *S. epidermidis* 33 GISK was associated with considerable adaptive modifications of their important physiological and morphological characteristics. The observed phenotypic impairments were accompanied by development of multiple cell resistances to antibacterial preparations. In our opinion, a promising approach for suppression of bacteria resistant to glycopeptide antibiotics includes the application of low-molecular-weight cationic peptides with lytic properties [34] as well as their combined use with other antibacterial preparations.

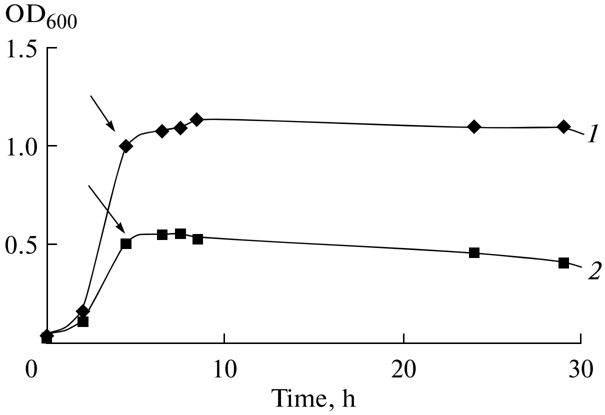

Fig. 6. Changes in optical densities of the cultures of *S. epidermidis* 33 GISK (*1*) and *S. epidermidis* 33 GISK Van^r (*2*) during their incubation with vancomycin. Arrows indicate the time of vancomycin addition.

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