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Low-Level Methicillin Resistance in Strains of *Staphylococcus aureus*

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Two strains of *Staphylococcus aureus* expressing borderline or low-level methicillin resistance by one or more in vitro test methods were examined for resistance in vivo and for biochemical and genetic markers of methicillin resistance. In vivo, nafcillin was equally effective against experimental aortic valve endocarditis in rabbits, regardless of whether they were infected by a fully susceptible or a low-level-resistant strain. Resistance did not emerge during therapy. For the more resistant of the two low-level-resistant strains, methicillin was as effective as nafcillin. Nafcillin was ineffective against endocarditis caused by a truly methicillin-resistant strain, and resistance emerged on therapy. The low-level-resistant strains did not produce the low-affinity penicillin-binding protein 2a that is associated with methicillin resistance and did not contain DNA that hybridized with probes that recognized the methicillin resistance determinant. Low-level resistance in *S. aureus* is a phenomenon that is biochemically and genetically distinct from true methicillin resistance. These strains actually are susceptible to beta-lactam antibiotics. The clinical problem posed by these strains is not a therapeutic one but, instead, one of how to differentiate them from those that are truly methicillin resistant.

Detection of methicillin-resistant strains of *Staphylococcus aureus* can be problematic, because resistance typically is heterogeneous, such that very few cells (as few as 1 in 10^5) express the trait (14). For this reason methicillin-resistant strains may appear to be susceptible to beta-lactam antibiotics in vitro unless susceptibility testing is modified to enhance expression of resistance. Well-standardized methods for the detection of methicillin-resistant *S. aureus* have been developed for the disk diffusion, broth dilution, and agar screening tests (20, 27).

Despite the best efforts and the use of recommended test methods, results with some strains may be indeterminant. Strains can appear to be susceptible in one test and have borderline or low-level resistance in others. It is not clear whether these indeterminant or low-level-resistant strains should be considered susceptible or resistant and what additional tests would be useful for determining susceptibility.

The purpose of this study was to determine whether two strains expressing low-level resistance in vitro were resistant in vivo. These strains were used to establish aortic valve endocarditis in rabbits, and the response of this infection to therapy with nafcillin or methicillin was measured. Both strains were examined for the presence of penicillin-binding protein (PBP) 2a or 2′ (4, 10–12, 28), which is uniquely associated with methicillin resistance in staphylococci. Their genomes were examined for hybridization with DNA probes that were specific for the methicillin resistance determinant, *mec*.

MATERIALS AND METHODS

Experimental strain. Strain 209P was a β-lactamase-negative, methicillin-susceptible strain that was obtained from the American Type Culture Collection (Rockville, Md.). Strain 1-63, a β-lactamase-producing, methicillin-susceptible strain, was a clinical isolate from a patient with endocarditis.

Strain VP-986 was a β-lactamase-producing clinical isolate that had intermediate susceptibility by the oxacillin disk test and was resistant by the broth dilution test (oxacillin MIC, 4 μg/ml). A β-lactamase-free clone was derived from strain VP-986 by treatment with ethidium bromide (2). Strain 29 was a β-lactamase-producing clinical isolate that expressed low-level methicillin resistance (oxacillin MIC, 16 μg/ml). A β-lactamase-free clone was also derived from strain 29.

Strain 67-0, both β-lactamase-producing and β-lactamase-free clones, was a methicillin-resistant strain that expressed heterogeneous resistance. Strain Col was a homogeneously resistant strain that was kindly provided by Alexander Tomasz.

Susceptibility studies. Disk diffusion tests were performed by the standardized methods recommended by Thornsberry and McDougal (27). A 1-μg oxacillin disk was used for disk diffusion tests, which were interpreted after incubation for 24 h at 35°C. No supplemental NaCl was added to the agar medium. A zone size of ≥13 mm was interpreted as indicating susceptibility.

MICs were determined by the microdilution method in cation-supplemented Mueller-Hinton broth plus 2% NaCl at an inoculum of approximately 5×10^5 CFU/ml (20, 27). MICs were interpreted after 24 h of incubation at 35°C. Breakpoints for susceptibility were ≤8 μg/ml for methicillin and ≤2 μg/ml for oxacillin and nafcillin.

Agar screening tests were performed as described by Thornsberry and McDougal (27), except that two inocula were used. Dilutions of 10^{-1} and 10^{-4} (corresponding to 10^7 to 10^8 and 10^4 to 10^5 CFU, respectively) from an overnight culture were inoculated onto Mueller-Hinton agar plus 4% NaCl containing 6 μg of oxacillin per ml, 6 μg of nafcillin per

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TABLE 1. Results of susceptibility studies

Strain	β-Lactamase production	Oxacillin disk zone (mm)[a]	MIC (μg/ml)[b]			Growth on agar screen for overnight culture at the indicated dilution[c]					
						Methicillin		Oxacillin		Nafcillin	
			Methicillin	Oxacillin	Nafcillin	10^{-1}	10^{-4}	10^{-1}	10^{-4}	10^{-1}	10^{-4}
209P	−	26 (S)	2	0.5	0.5	+	−	+	−	+	−
1-63	+	18 (S)	4	1	1	+	−	+	−	+	−
VP-986	−	14 (S)	4	1	0.5	+	−	+	−	+	−
	+	12 (I)	4	4	4	+	−	+	−	+	−
29	−	No zone (R)	8	16	8	+	+	+	+	+	−
	+	No zone (R)	16	16	4	+	+	+	+	+	−
67-0	−	No zone (R)	>64	>64	64	+	+	+	+	+	+
	+	No zone (R)	>64	>64	>64	+	+	+	+	+	+

[a] Abbreviations: S, Susceptible (zone size, ≥13 mm); I, intermediate (zone size, 11 to 12 mm); R, resistant (zone size, ≤11 mm).
[b] Results shown are for cation-supplemented Mueller-Hinton broth plus 2% NaCl.
[c] Methicillin was used at a concentration of 10 μg/ml, oxacillin at 6 μg/ml, and nafcillin at 6 μg/ml in Mueller-Hinton agar plus 4% NaCl. Results were interpreted after 24 h at 35°C for 10^{-1} (10^7 to 10^8 CFU) and 10^{-4} (10^4 to 10^5 CFU) dilutions from a 1-ml overnight culture. +, Growth of one or more colonies; −, no growth.

ml, or 10 μg of methicillin per ml and were incubated at 35°C for 24 h. Growth of the more dilute sample was interpreted as being resistant.

Rabbit endocarditis model. Endocarditis was established in New Zealand White rabbits (weight, 2 kg) (21). A catheter was positioned across the aortic valve and secured in place for the duration of the experiment. Twenty-four hours later, 1 ml of approximately 10^7 CFU in 0.9% saline was injected intravenously. Twenty-four hours later, treatment with nafcillin (100 mg/kg every 8 h intramuscularly) or methicillin (75 mg/kg every 6 h intramuscularly) was begun, and the antibiotics were administered for a total of 4 days.

Peak drug concentrations in serum were determined from blood samples that were obtained 1 h after dosing, usually on day 2 of therapy. Trough concentrations were determined in samples that were obtained 6 h after administration of a methicillin dose and 8 h after administration of a nafcillin dose. Concentrations in serum were measured by an agar diffusion assay (23).

Rabbits were sacrificed 12 h after they received the last dose. Aortic valve vegetations were removed, homogenized in 0.5 ml of 0.9% NaCl, and quantitatively subcultured onto blood agar to determine the total number of organisms remaining in the vegetation. To test for resistant cells, samples, including 0.1 ml of undiluted homogenate, were also inoculated onto Trypticase soy agar (TSA; BBL Microbiology Systems, Cockeysville, Md.) containing either 5 μg of nafcillin per ml or 10 μg of methicillin per ml and were incubated for 48 h at 37°C. The number of organisms in the vegetation was expressed as the vegetation titer, which was defined as the $\log_{10}$ CFU per gram of vegetation.

For each strain the mean vegetation titer of untreated rabbits was compared with that of treated rabbits. Statistical significance, defined as $P < 0.05$, was determined by either Student's *t* test (for two groups) or analysis of variance (for more than two groups).

PBP studies. Exponentially growing cells in Trypticase soy broth (BBL) plus 4% NaCl were harvested at a cell density of 2×10^8 to 5×10^8 CFU/ml, washed, and then mechanically disrupted. Membranes were separated by differential centrifugation and suspended in 10 mM Tris hydrochloride (pH 7.0) to a final protein concentration of 10 mg/ml, as assayed with a protein assay kit (Bio-Rad Laboratories, Richmond, Calif.) (3). Samples of 30 μg of membrane protein were preincubated either with buffer (direct PBP assay) or antibiotic for 15 min at 37°C. [^{3}H]penicillin (20 μg/ml) was then added for a 15-min incubation at 37°C. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% acrylamide, 0.13% bisacrylamide) (15) and stained with Coomassie blue. Radiolabeled proteins were detected by fluorography with prefogged film (X-Omat; Eastman Kodak Co., Rochester, N.Y.) (16). Binding of [^{3}H]penicillin to PBPs was measured by scanning densitometry (Quick-Scan, Jr., Helena Instruments), and the affinity of nafcillin and methicillin for PBPs was expressed in terms of the concentration at which a 50% or greater decrease in band density occurred.

DNA hybridization studies. Probes A and B were derived from different regions of the cloned PBP 2a gene from the methicillin-resistant strain TK784 (18). Probe A was a 0.6-kilobase (kb) *Hin*cII-*Sau*3A fragment of the promoter region, and probe B was a 0.8-kb *Hae*III-*Cla*I fragment derived from the PBP gene (25).

Probe pG0158 was a 1.3-kb *Pst*I-*Bgl*II fragment, and pG0159 was a contiguous 3.9-kb *Hin*dIII fragment; both of these probes were derived from DNA that was unique to the methicillin resistance gene, *mec*. They were initially cloned from the Col strain of *S. aureus* and shown to be specific for methicillin-resistant staphylococci by hybridization with a panel of resistant and susceptible isolates (D. Galetto, J. Froggatt, J. Kornblum, and G. Archer, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, A59, p. 10). By comparison of the restriction endonuclease cleavage site map for these probes with published sequence data for PBP 2a (25), they probably contain the PBP 2a gene plus more than 1 kb each of 3′- and 5′-flanking sequences (G. Archer, manuscript in preparation). DNA preparation and electrophoresis were done as described previously (9, 17, 24). DNA transfer and hybridization were done by the technique of Southern (26) under conditions of high stringency (9).

RESULTS

Susceptibility studies. Strains 209P and 1-63 were susceptible to methicillin, oxacillin, and nafcillin by the disk diffusion and brothdilution methods (Table 1). At the lower inoculum of approximately 10^4 to 10^5 CFU, these strains were susceptible by the agar screening method. However, at the higher inoculum of 10^7 to 10^8 CFU, a zone of hazy growth was observed at the site of inoculation.

The β-lactamase-positive strain VP-986 was intermediate in the oxacillin disk test (zone size, 12 mm), susceptible to

TABLE 2. Mean bacterial counts in aortic valve vegetations of rabbits

Strain	Mean bacterial count (log_{10} CFU/g) after treatment with:		
	No treatment	Nafcillin[a]	Methicillin[b]
1-63	7.3 ± 2.3 (4)[c]	3.4 ± 2.1 (7)	ND[d]
VP-986	7.6 ± 2.9 (5)	3.2 ± 2.7 (10)	ND
29	7.0 ± 1.2 (6)	3.3 ± 1.0 (6)	3.1 ± 1.6 (6)
67-0	8.7 ± 0.8 (5)	6.6 ± 3.3 (7)	ND

[a] Nafcillin (100 mg/kg) was given every 8 h intramuscularly for 4 days.
[b] Methicillin (75 mg/kg) was given every 6 h intramuscularly for 4 days.
[c] Numbers in parentheses are the number of rabbits in each group.
[d] ND, Not done.

methicillin (MIC, 4 μg/ml), but resistant to nafcillin and oxacillin by the microdilution method. At a low inoculum this strain was susceptible to all three antibiotics by the agar screening method. The β-lactamase-negative clone of strain VP-986 was susceptible to the three antibiotics by all three methods.

Strain 29 was resistant to oxacillin in the disk test; to methicillin, oxacillin, and nafcillin in the microdilution test; and to methicillin and oxacillin in the agar screening test. It did not grow at the lower inoculum in the agar screening test with nafcillin. The β-lactamase-negative clone had susceptibilities similar to those of the β-lactamase-positive strain.

Endocarditis studies. Nafcillin concentrations in serum were 12 ± 8 μg/ml (n = 6) 1 h after a dose and 0.6 ± 0.6 μg/ml (n = 6) 8 h after a dose. Methicillin concentrations were 15 ± 7 μg/ml (n = 5) 1 h after a dose and were undetectable at 6 h after a dose.

For the three susceptible or low-level-resistant strains, the mean vegetation titers were virtually identical in untreated rabbits that were sacrificed 24 h after infection, ranging from 7.0 to 7.6 log_{10} CFU/g (Table 2). For the resistant strain 67-0, the mean titer of 8.7 log_{10} CFU/g at the start of therapy was slightly higher than those for the other strains, but the difference did not achieve statistical significance ($0.05 < P < 0.1$). No resistant CFU (i.e., growth of colonies on TSA containing either 5 μg of nafcillin per ml or 10 μg of methicillin per ml) were detected in untreated rabbits infected by susceptible or low-level-resistant strains. For the resistant strain 67-0, vegetations from three untreated rabbits that were tested contained CFU at a rate of approximately 1 in 5,000 CFU that would grow on TSA containing 10 μg of nafcillin per ml.

After 4 days of nafcillin therapy, mean bacterial counts were similar for the three susceptible or low-level-resistant strains, ranging from 3.2 to 3.4 log_{10} CFU/g ($P < 0.01$ for nafcillin-treated rabbits versus no treatment for each strain). No resistant colonies grew on TSA containing 5 μg of nafcillin per ml. Vegetation titers from rabbits infected with strain 29, which was the most resistant to methicillin (MIC, 16 μg/ml), and treated with methicillin were the same as those from nafcillin-treated rabbits. No colonies grew from vegetations that were cultured onto TSA containing 10 μg of methicillin per ml.

For rabbits infected with the resistant strain 67-0, after 4 days of nafcillin therapy, mean vegetation titers were 6.6 ± 3.3 log_{10} CFU/g ($0.05 < P < 0.1$ compared with the control). This mean value was skewed by a single rabbit with a sterile vegetation. When results for this rabbit were excluded from the analysis, the mean was 7.6 ± 1.8 log_{10} CFU/g. Over the course of therapy, the proportion of resistant CFU (i.e., CFU growing on TSA containing 10 μg of nafcillin per ml) in the vegetations increased 1,000-fold from 1 resistant CFU in 5,000 in untreated rabbits to 1 in 5 in treated rabbits ($P < 0.002$).

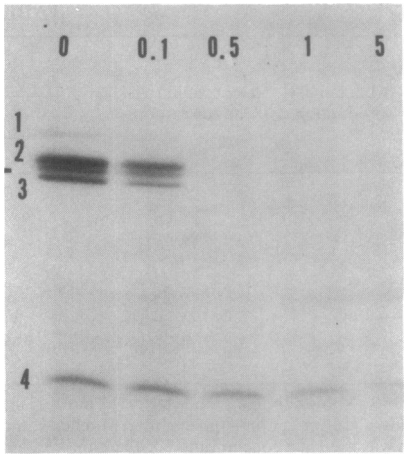

FIG. 1. Assay of PBPs in membrane samples of the low-level methicillin-resistant strain 29. Numbers at the left indicate PBPs; the dash indicates a novel PBP that migrated between PBPs 2 and 3. The numbers at the top indicate the concentration (in micrograms per milliliter) of nafcillin used in the competition assay to determine PBP-binding affinity.

PBP studies. The 78-kilodalton low-affinity PBP 2a was not detected in either strain 29 (Fig. 1) or strain VP-986 (data not shown). Growth of cells in medium containing 5 μg of nafcillin per ml did not induce PBP 2a in either strain. For all strains, PBPs 1, 2, and 3 were >50% saturated at nafcillin concentrations of 0.5 μg/ml and above and PBP-4 was >50% saturated at concentrations of 1 to 5 μg/ml and above (Table 3). For strain 29 PBPs 1 and 3 were >50% saturated at a methicillin concentration of 0.5 μg/ml and PBPs 2 and 4 were >50% saturated at a methicillin concentration of 5 μg/ml (data not shown).

Strain 29 did not produce a low-affinity PBP 2a, but it did produce an extra PBP that migrated between PBP 2 and PBP 3 (Fig. 1). This extra PBP, although similar in molecular size to the 78-kilodalton PBP 2a produced by methicillin-resistant strains, differed in that it had a high affinity for nafcillin and was completely saturated at a concentration of 0.5 μg/ml.

DNA hybridization studies. DNA probes specific for the methicillin resistance determinant, *mec*, hybridized with DNA digests of methicillin-resistant strains 67-0 and Col

TABLE 3. Saturation of PBPs in the experimental *S. aureus* strains

Strain	Phenotype[a]	Nafcillin concn (μg/ml) producing ≥50% saturation of the following PBPs binding for [^{3}H]penicillin:				
		PBP 1	PBP 2	PBP 2a[b]	PBP 3	PBP 4
209P	Susceptible	0.5	0.5	—	0.5	5
VP-986	Lo-MR	0.5	0.5	—	0.5	1
29	Lo-MR	0.5	0.5	0.5	0.5	1
67-0	Het	0.5	0.5	500	0.5	5

[a] Lo-MR, Low-level resistance; het, heterogeneous methicillin resistance.
[b] —, Not detectable; strain 29 did not produce a low-affinity PBP 2a but had a high-affinity PBP that migrated in the same position as PBP 2a in sodium dodecyl sulfate-polyacrylamide gels.

TABLE 4. Hybridization of *mec*-specific DNA probes in resistant and low-level-resistant strains

Strain	Endonuclease digestion	Hybridization of the following probes[a]:			
		A	B	pG0158	pG0159
63	*Hin*dIII	–	–	ND	ND
	*Hin*dIII-*Hae*III	–	–	ND	ND
VP-986	*Bgl*II	ND	ND	–	–
	*Hin*dIII	–	–	–	–
	*Hin*dIII-*Hae*III	–	–	ND	ND
29	*Bgl*II	ND	ND	–	–
	*Hin*dIII	–	–	–	–
	*Hin*dIII-*Hae*III	–	–	ND	ND
67-0	*Bgl*II	ND	ND	+ (2.3)	+ (2.3, 3.0)
	*Hin*dIII	+ (4.0)	+ (4.0)	+ (0.8, 3.8)	+ (3.8)
	*Hin*dIII-*Hae*III	+ (1.9)	+ (2.1)	ND	ND
Col	*Hin*dIII	+ (4.0)	+ (4.0)	+ (1.3, 3.8)	+ (3.8)
	*Hin*dIII-*Hae*III	ND	+ (2.1)	ND	ND

[a] Abbreviations and symbols: ND, not done; a plus sign indicates hybridization, and the value in parentheses is the size of the fragment (in kilobases); a minus sign indicates that no hybridization was detected.

(Table 4). These probes did not hybridize with DNA of the susceptible strain 1-63 or with DNA of strains 29 or VP-986.

DISCUSSION

Two strains expressing low-level methicillin resistance were examined for evidence of resistance in vivo in an experimental endocarditis model. The presence of PBP 2a, the biochemical marker of methicillin resistance, was also sought, and four different DNA probes to detect the methicillin resistance determinant were used.

In vivo, biochemically, and genetically, both low-level-resistant strains were susceptible. Nafcillin and methicillin significantly reduced vegetation titers in vivo, and resistant subpopulations did not emerge during therapy. The response to therapy for these strains was like that for the fully susceptible strain.

In contrast, nafcillin did not significantly reduce the titers in the rabbits infected with the truly methicillin-resistant strain 67-0, and the proportion of resistant CFU present in the vegetations increased markedly. These results are very similar to those from an earlier study in which cephalothin, with an MIC lower than that for nafcillin, also was ineffective for experimental endocarditis caused by strain 67-0 (7).

Unlike truly resistant strains, neither low-level-resistant strain produced PBP 2a. Likewise, susceptible and low-level-resistant strains did not hybridize with DNA probes which detected the methicillin resistant determinant in truly resistant strains.

In vitro tests misclassified these strains as resistant. The best way to determine in the clinical laboratory whether a strain expressing low-level resistance is susceptible or resistant is not known. Genetic probes for the methicillin resistance determinant or detection methods for PBP 2a are attractive possibilities, but the technology does not exist to permit these tests to be performed rapidly, as would be necessary in the clinical laboratory.

A property that might be useful clinically in determining whether a strain is resistant is the presence of a highly resistant subpopulation and its emergence on exposure to beta-lactam antibiotics. The presence of a resistant subpopulation was recognized in the very first methicillin-resistant strains of *S. aureus* (14). These highly resistant cells grew in concentrations of ≥50 μg of methicillin per ml. The resistant subpopulation can be readily selected from a heterogeneous strain by passage on agar containing a penicillinase-resistant penicillin. Unlike the heterogeneous parent strain, these resistant cells produce homogeneously resistant progeny which can grow uniformly at high concentrations of drug, e.g., 500 μg of methicillin per ml, even without special culture conditions.

When susceptible or low-level-resistant (but truly susceptible) strains such as 29 or VP-986 are plated onto agar containing methicillin, oxacillin, or nafcillin lacking NaCl supplementation, no colonies grow above a concentration of 1 μg/ml after incubation for 48 h at 35°C. If the few colonies that grew at 0.5 μg/ml were then subcultured onto agar containing 10 or 50 μg of antibiotic per ml and reincubated, no growth of these subcultures occurred. If 4% NaCl was added to the agar, some colonies grew at concentrations of 6 to 10 μg/ml, but these could not be passed to the same or a higher concentration if the salt was removed from the medium. Several attempts to select out highly resistant subpopulations from strains 29 or VP-986 by repeated passages in either broth or on agar containing nafcillin failed.

The resistant subpopulation of a truly methicillin-resistant heterogeneous strain was readily identified by passage in antibiotic. Perhaps confirmatory tests based on the selection of resistant subpopulations that can grow in the presence of high antibiotic concentrations would be a useful way to determine whether strains expressing low-level resistance are susceptible or resistant.

The mechanisms responsible for low-level resistance remain to be defined. β-Lactamase production has been suggested as a possible mechanism (19). Results with the β-lactamase-negative variant of strain VP-986 are evidence that β-lactamase production can be important. The β-lactamase-producing isolate was borderline resistant by the oxacillin disk method and resistant (MIC, 4 μg/ml) to nafcillin and oxacillin by the microdilution method, whereas the strains cured of β-lactamase were susceptible to each antibiotic by all three methods.

The culture conditions used to enhance the expression of methicillin resistance may promote β-lactamase production or activity (8, 13) with hydrolysis of penicillinase-resistant penicillins, so that a higher concentration is required to inhibit growth of the strain. That the addition of the β-lactamase inhibitor clavulanic acid to penicillinase-resistant penicillins causes some indeterminant or borderline resistant strains to be susceptible is further evidence that β-lactamase plays a role in low-level resistance (19).

Results with strain 29 indicate that mechanisms other than β-lactamase production can also produce low-level resistance. Results of susceptibility tests for strain 29 were identical for β-lactamase-producing and β-lactamase-free clones.

It is intriguing to speculate that the extra PBP identified in strain 29 has a role in the expression of low-level resistance. This PBP has not been described in other susceptible strains. Unlike PBP 2a, it has a high binding affinity for beta-lactam antibiotics. Whether this PBP is unique or a degradation or a related product of another PBP (PBP 2 perhaps) remains to be determined.

Another possible mechanism for low-level resistance is that the autolytic system may be altered somehow, as has been suggested for truly methicillin-resistant strains (5, 6, 22). Perhaps, in the presence of NaCl or a lower temperature of incubation, these strains can survive concentrations of beta-lactam antibiotics above the conventional breakpoints for susceptibility.

Whatever the mechanism(s) of the in vitro phenomenon of low-level resistance proves to be, resistance is probably an artifact of the test system and is not clinically important. Strains expressing low-level resistance appeared to be fully susceptible in vivo and had none of the markers of methicillin-resistant strains. Infections caused by these strains probably can be safely and effectively treated with beta-lactam antibiotics. The clinical problem remains of how best to determine whether a particular strain expressing low-level resistance is susceptible or methicillin resistant.

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