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Drug Resistance of Staphylococci

VI. Genetic Determinant for Chloramphenicol Resistance

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Naturally occurring strains of staphylococci which are resistant to chloramphenicol (CM) inactivate this antibiotic. One of the inactivation products of CM showed the chromatographic behavior of 3-acetoxychloramphenicol. Induction of resistance occurred after prior exposure to subinhibitory concentrations of the antibiotic. The resistance of induced populations, as well as CM-inactivation ability, was decreased when they were grown in CM-free medium. The CM-inactivation property was transduced together with CM resistance. Transductional analysis and CM-resistance elimination experiments indicated that CM resistance in naturally occurring strains of staphylococci is mainly accounted for by inactivation of the drug.

Surveys of microbial drug resistance have revealed that resistance to chloramphenicol (CM) in strains of *Staphylococcus aureus* and in gram-negative bacteria such as *Shigella*, *Escherichia coli*, *Proteus mirabilis*, and the *Klebsiella-Aerobacter* group has been increasing. This resistance has become a serious clinical problem (5, 9). It has been found recently that CM resistance in gram-negative bacteria derived from clinical sources is due mainly to the presence of the episomal resistance transfer factor, R (7, 8, 10, 18), and that such strains inactivate the antibiotic by enzymatic acetylation (16, 17). Similarly, Suzuki, Okamoto, and Kono (19) found that naturally occurring strains of staphylococci resistant to CM inactivate it by acetylation. However, the genetics of CM resistance in such strains has not been fully investigated.

This paper deals with genetic analysis of the determinant of CM resistance and the detection of the inactivated products of CM in strains of *S. aureus* isolated from clinical sources.

MATERIALS AND METHODS

Bacterial strains. Coagulase-positive staphylococcal strains were isolated from patients at hospitals in Japan. Strains resistant to CM were selected at random from these cultures. *S. aureus* 209P, which is susceptible to CM, was employed as reference strain for antibiotic resistance. *S. aureus* 209P CM^r, resistant to 25 μg of CM/ml, was obtained by subculturing 209P on plates containing serially increasing amounts of CM and was maintained in a medium containing 12.5 μg of CM/ml. *Bacillus subtilis*, which is sensitive to CM, was used for bioassay of this drug.

Media. Heart infusion (HI)-agar (Eiken, Tokyo) was used for the determination of drug resistance. Penassay Broth (Difco) was used routinely for the propagation of bacteria. Nutrient agar was used for the bioassay of CM. It consisted of 10.0 g of beef extract (Difco), 10.0 g of polypetone (Daigo Eiyo Co.), 2.0 g of sodium chloride, 17.0 g of agar, and 1,000 ml of deionized water. The *p*H was adjusted to 6.5. Brain Heart Infusion (BHI; Difco), adjusted to *p*H 7.6, was used to study the elimination of resistance to CM by acriflavine treatment. Soft agar consisted of 1,000 ml of BHI and 8.5 g of agar.

Determination of bacterial growth. Growth in liquid culture was assayed turbidimetrically at 600 mμ. A standard curve was constructed by plotting the dry weight of bacteria versus optical density at 600 mμ.

Inactivation of CM. Organisms to be tested were inoculated in 10.0 ml of Penassay Broth containing 5.0 μg of CM/ml and incubated at 37 C. After incubation for 18 hr, the culture was harvested by centrifugation and suspended in 1 ml of fresh medium containing 500 μg of CM. After incubation at 37 C in a water bath without shaking, the reaction mixture was heated at 95 C for 5 min and then chilled in ice water. After centrifugation at 2,000 × *g* for 10 min, the residual CM in the supernatant fluid was determined by bioassay. CM inactivation is expressed as micrograms of CM inactivated by 1 mg (dry weight) of bacteria in 1 hr.

Determination of drug resistance. The agar dilution method used was described previously (13).

Bioassay of CM. A 0.1-ml portion of an overnight broth culture of *B. subtilis* was mixed with 3.0 ml of melted soft agar and overlayered onto 20 ml of nutrient agar prewarmed to 37 C. Stainless-steel cups were set on the agar medium, and 0.2 ml of a solution to be tested was poured into each cup. After being in a refrigerator for 2 hr, the agar plate was incubated

at 37 C. After 24 hr of incubation, the diameter of the inhibition zone on the bacterial lawn was scored and the concentration of CM was calculated from a standard curve prepared with standard solutions of CM. Deviation among duplicate determinations was generally less than ±5%.

Drugs. The drugs used were benzylpenicillin (PC), dihydrostreptomycin (SM), tetracycline (TC), CM, erythromycin, and oleandomycin, supplied by H. Umezawa, National Institute of Health, Japan. A chromatographically pure sample of leucomycin A was supplied by Z. Abe, The Toyo Jozo Co., Tokyo. 3-Acetoxychloramphenicol and 1,3-diacetoxychloramphenicol were supplied by I. Iwai, The Sankyo Pharmaceutical Co., Tokyo. Mitomycin C was supplied by The Kyowa Hakko Kagaku Co., Tokyo. D-Threo-chloramphenicol(methylene-^{14}C) was purchased from The Radiochemical Centre, Amersham, Backinghamshire, England.

Identification of inactivated products of CM. Organisms were inoculated in 10.0 ml of Penassay Broth containing a subinhibitory concentration of CM (5.0 μg/ml) and incubated at 37 C. After incubation for 18 hr, the culture was harvested by centrifugation and suspended in 1 ml of fresh medium containing ^{14}C-labeled CM (30,000 counts/min). After overnight incubation at 42 C, the bacteria were removed by centrifugation and the supernatant fluid was heated at 95 C for 5 min. The supernatant liquid thus obtained was extracted with 3 ml of cold ethyl acetate. After vigorous shaking, followed by centrifugation, the layer of ethyl acetate was removed. The extraction was repeated twice and the combined ethyl acetate fractions were evaporated to dryness at room temperature. The residue was dissolved in one drop of ethyl acetate and spotted onto Toyo Roshi no. 51A paper, and ascending chromatography was carried out in benzene, methanol, and water (96:2:2). The chromatogram was assayed by scanning with an Aloka scanner (12.5 mm/min, 10-sec response).

For the identification of inactivated products of CM, the bacteria harvested as described above were inoculated in 1 ml of fresh broth containing CM (100 μg/ml). After 10 min of incubation at 37 C with gentle shaking, the culture was heated at 95 C for 5 min and the bacteria were removed by centrifugation. The supernatant liquid was then extracted with 3 ml of ethyl acetate. After vigorous shaking, followed by centrifugation, the layer of ethyl acetate was removed. This extraction was repeated twice and the combined ethyl acetate fractions were dried in an air stream at room temperature. The residue was dissolved in one drop of ethyl acetate and spotted on a thin-layer plate of silica gel (Kieselgel H, E. Merck AG, Darmstadt, Germany); ascending chromatography was carried out in benzene-*n*-butanol (90:10), *n*-butanol-chloroform-acetic acid (10:89.5:0.5), or chloroform-methanol (95:5). The chromatograms were developed with 0.25% stannous chloride in 1 N HCl after treatment with zinc dust and with 2% *p*-dimethylaminobenzaldehyde in 1.2 N HCl-alcohol. Reduced nitro compounds appeared as yellow spots.

Elimination of CM resistance. The organism to be tested was subjected to three successive single-colony isolations and inoculated to BHI. An overnight culture was diluted 1:100 with fresh medium, and 0.1 ml of the diluted culture was inoculated into a series of 10-ml tubes of BHI containing serial twofold dilutions of acriflavine. After incubation for 18 hr at 37 C, the culture containing the maximum concentration of acriflavine allowing growth was used to test for elimination of resistance. An appropriate dilution of such a culture was spread on an agar plate, which was used as the master plate for examination of elimination of CM resistance. This examination was accomplished by replica-plating onto a medium containing CM (12.5 μg/ml). Loss of resistance in each colony was confirmed by determination of CM resistance by the agar plate method after two successive single-colony isolations on plates without CM.

Transduction of resistance to CM. Transduction of resistance to CM was carried out as described previously (13). *S. aureus* MS353 and S1396 were used as the recipients of CM resistance. An overnight broth culture of the donor of CM resistance was diluted 1:100 with fresh broth containing mitomycin C (1.0 μg/ml) and aerated on a shaking machine at 37 C. After 3 hr of incubation, the resulting phage lysate from the donor strain was clarified by centrifugation at 10,000 × *g* for 10 min and filtered through a membrane filter (HA; Millipore Corp., Bedford, Mass.). An overnight broth culture of the recipient organism was diluted 1:100 with fresh broth and incubated at 37 C with shaking. After 4 hr of incubation, 0.5 ml of the recipient culture was mixed with an equal volume of the phage lysate of the donor strain. After 60 min of incubation at 37 C, 0.1 ml of an appropriately diluted mixture was spread on an HI-agar plate containing CM (12.5 μg/ml). After incubation for 48 hr, the colonies which developed on the selective plate were picked and subjected to two successive single-colony isolations. Their drug resistance and phage type were then determined. As controls in the transduction experiment, a sterility test of the phage lysate and a test for mutation of the recipient organism without phage lysate were conducted in every experiment.

RESULTS

Inactivation of CM. Naturally occurring strains of *S. aureus* resistant to CM were tested for CM inactivation; 34 inactivated the antibiotic. By contrast, 11 CM-susceptible strains, *S. aureus* 209P CM^s and 209P CM^r, were incapable of inactivating the antibiotic during overnight incubation at 37 C. The CM-resistance property in naturally occurring strains of staphylococci was eliminated by treatment with acridine (Table 1). CM resistance of the strains resistant to CM was lost at relatively high frequency by treatment with acridine dye, as compared with the frequency of spontaneous loss of CM resistance in the same culture. The strains from which the CM resistance property was eliminated have not so far been observed to revert to CM resistance, indicating the irreversible elimination of CM resistance.

TABLE 1. *Elimination of CM resistance in Staphylococcus aureus*[a]

Strain	Without acriflavine treatment		With acriflavine treatment	
	No. of colonies tested	No. of colonies from which CM resistance was lost	No. of colonies tested	No. of colonies from which CM resistance was lost
S1337	520	1 (0.1)	570	24 (4.1)
S1477	298	1 (0.3)	594	20 (3.4)
S1484	483	2 (0.4)	426	9 (2.1)

[a] The strains were of clinical origin and resistant to CM. Numbers in parentheses indicate percentages of colonies losing CM resistance.

Naturally occurring strains of staphylococci carrying CM resistance were consistently multiply resistant to TC, sulfanilamide, SM, PC, and sometimes to macrolide antibiotics (9). However, the elimination of CM resistance was not accompanied by elimination of the determinant for penicillinase formation or of the genetic characters responsible for resistance to TC, SM, sulfanilamide, and macrolide antibiotics (Table 2). CM inactivation was then investigated by detection of acetylated products of ^{14}C-labeled CM in the strains from which CM resistance was lost. Two radioactive peaks were seen when CM was inactivated by CM-resistant strains. One was noted at the origin and corresponded to CM; the other was noted at an R_F value of 0.95, corresponding to that of 3-acetoxychloramphenicol. By contrast, only one peak was noted at the origin with CM after exposure to CM-susceptible strains obtained by acriflavine treatment. The property of CM inactivation was lost together with the loss of CM resistance (Table 2).

Induction of resistance to CM. It was noticed that faint growth occurred on the plates containing high concentrations of CM after long periods of incubation. It was observed also that CM-resistant cells grown in the presence of subinhibitory concentrations of CM showed much more inactivation than did those grown in its absence (19). The effect of varying the CM concentration in broth on the growth of an inducible strain (S1477) is shown in Fig. 1. Bacterial cells in early exponential phase were inoculated in broth containing various concentrations of CM and incubated with shaking. At 6.25 μg of CM/ml, growth was as rapid as in CM-free broth. At concentrations of 12.5 and 25 μg of CM/ml, the lag time was prolonged; growth was completely inhibited at 50 μg of CM/ml.

The induced populations at various concentrations of CM showed varied growth in broth containing 50 μg of CM/ml. The induced populations in broth containing 2.5, 5.0, and 10 μg of CM/ml showed almost the same growth as that obtained in CM-free medium, indicating that these concentrations of CM were sufficient to complete the induction of resistance under the conditions described (Fig. 2).

TABLE 2. *Loss of CM-inactivating capacity in Staphylococcus aureus after loss of CM resistance*

Strain	Drug resistance[a]						Inactivation of CM[b]
	CM	TC	SM	PC	SA	Mac	
S1337	r	r	r	r	r	r	+
S1337-1	s	r	r	r	r	r	−
S1337-3	s	r	r	r	r	r	−
S1337-5	s	r	r	r	r	r	−
S1477	r	r	s	r	r	s	+
S1477-1	s	r	s	r	r	s	−
S1477-3	s	r	s	r	r	s	−
S1477-5	s	r	s	r	r	s	−
S1484	r	s	s	r	r	s	+
S1484-1	s	s	s	r	r	s	−
S1484-3	s	s	s	r	r	s	−
S1484-5	s	s	s	r	r	s	−

[a] Abbreviations: CM, chloramphenicol; TC, tetracycline; SM, dihydrostreptomycin; PC, benzylpenicillin; SA, sulfanilamide; Mac, macrolide antibiotics (erythromycin, oleandomycin, and leucomycin); r, resistant; s, susceptible.

[b] As detected in the form of acetylated products of ^{14}C-labeled CM.

Induction of resistance to high concentrations of CM occurred after prior exposure to subinhibitory concentrations of CM (Table 3). CM inactivation increased in the induced populations. However, the CM resistance of induced populations was decreased when they were grown in the absence of this antibiotic, as was the capacity to inactivate CM. Detailed studies of induction of CM resistance will be described elsewhere.

Transduction of CM resistance. CM resistance was transduced to recipient strains MS353 and S1396 at relatively high frequency (Table 4). The CM-resistant transductants acquired the ability to inactivate CM.

Detection of inactivated products of CM. Suzuki, Okamoto, and Kono (19) suggested that extracts of naturally occurring strains of staphylococci resistant to CM contain an enzyme(s) which inactivates CM through acetylation of the molecule and that the product of CM acetylation is 3-acetoxychloramphenicol (Okamoto, *unpublished data*).

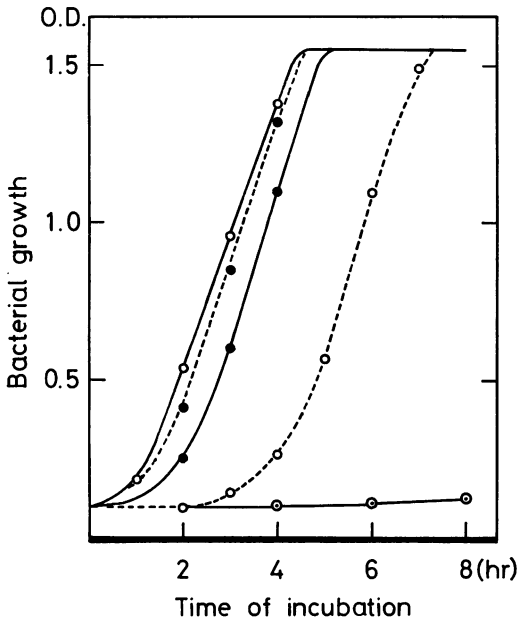

FIG. 1. *Growth of an inducible strain in broth containing various concentrations of CM. An overnight culture of S1477 was diluted 10-fold with fresh broth and shaken at 37 C for 2 hr; 0.3 ml of the culture in early phase of exponential growth was inoculated in 10 ml of broth containing various concentrations of CM and shaken at 37 C. Bacterial growth was determined photometrically.* (○—○) *CM-free control;* (●--●) *6.25 μg of CM/ml;* (●—●) *12.5 μg of CM/ml;* (○--○) *25 μg of CM/ml;* (⊙) *50 μg of CM/ml.*

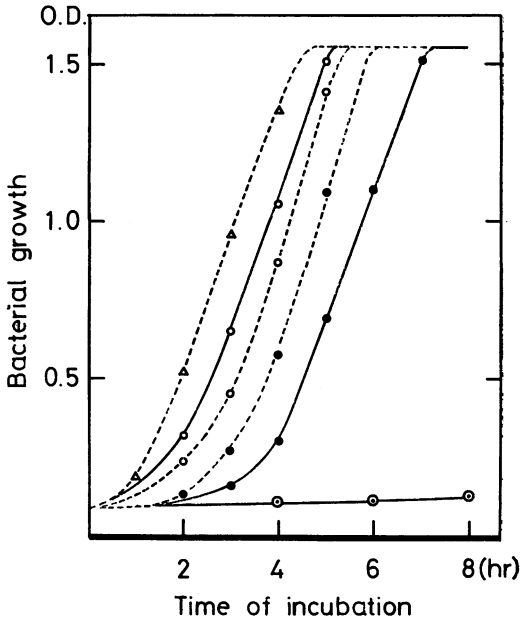

FIG. 2. *Growth of induced populations in broth containing 50 μg of CM/ml. An overnight culture of S1477 was diluted 10-fold with fresh broth and shaken at 37 C for 2 hr; 0.3 ml of the culture in early phase of exponential growth was inoculated in 10 ml of broth containing various concentrations of CM and shaken at 37 C. After 2 hr of incubation, the culture was diluted with fresh broth and the induced culture was inoculated in 10 ml of broth containing 50 μg of CM/ml and shaken at 37 C. Bacterial growth was determined photometrically.* (⊙) *CM-free;* (●—●) *1.25 μg of CM/ml;* (○--○) *2.5 μg of CM/ml;* (○—○) *5.0 μg of CM/ml;* (●--●) *20 μg of CM/ml (10 μg of CM/ml was almost the same as 2.5 μg of CM/ml);* (△) *normal growth of the strain.*

To examine this suggestion, the products formed by inactivation of CM with S1477 CM^r were extracted with ethyl acetate, and ascending chromatography was carried out with thin-layer plates. Under these conditions, one major product and two minor components showing faint density were demonstrated (Table 5). From chromatographic behavior, it seemed likely that a major product (c) was 3-acetoxychloramphenicol. A minor component (a) showing a low R_F value was found to correspond to noninactivated CM. Another component (b), showing faint density, was not identified. 1,3-Diacetoxychloramphenicol was not detected under these conditions.

DISCUSSION

The determinant of resistance to macrolide antibiotics in staphylococci is located in a single genetic determinant, a plasmid which exists extrachromosomally (6, 11, 12). Similarly, it was found that the genetic determinant of penicillinase formation in *S. aureus* is incorporated in an extrachromosomal element or plasmid (4, 14, 15). Thus, three different plasmids have been described: one carries only a genetic determinant for penicillinase formation, one carries the genetic determinants of both penicillinase formation and resistance to mercuric ion, and one governs penicillinase formation and resistance to macrolide antibiotics (4, 14, 15).

Resistance to CM is labile in some strains of staphylococci and is eliminated irreversibly on aging or by treatment with acridine (1). Similarly, resistance to CM in the strains used in the present study was also eliminated both spontaneously and by treating them with acriflavine. However, the genetic determinant responsible for CM resistance and the reasons for the instability of such resistance were not fully investigated; whether the determinant for labile CM resistance is located on a plasmid remains to be determined.

TABLE 3. *Effect of CM on inducible resistance to CM*

Organism[a]	Before treatment	Resistance to CM (μg/ml)		
		After treatment with[b]		After overnight incubation of CM-treated cells in CM-free medium[c]
		2.5 μg of CM/ml	5.0 μg of CM/ml	
S1337 CM^r	25 (30)[d]	200 (1,600)	200 (1,800)	25 (30)
S1477 CM^r	25 (20)	100 (1,000)	200 (2,500)	25 (30)
S1484 CM^r	25 (20)	100 (1,600)	200 (1,000)	25 (25)
MS636 CM^r	25 (30)	200 (1,800)	200 (1,000)	25 (30)
S1343 CM^s	3.2	3.2	3.2	3.2
S1556 CM^s	3.2	3.2	3.2	3.2
S1574 CM^s	3.2	3.2	3.2	3.2
S2126 CM^s	3.2	3.2	3.2	3.2
MS353 CM^s	1.6	1.6	1.6	1.6
209P CM^s	1.6	1.6	1.6	1.6

[a] Naturally occurring strains of staphylococci resistant (CM^r) and susceptible to (CM^s) CM; 209P is a reference strain used for determining antibiotic resistance.

[b] An organism to be tested was inoculated in Penassay Broth containing CM (2.5 or 5.0 μg/ml) and incubated at 37 C. After overnight incubation, the bacteria were harvested by centrifugation, suspended in fresh medium, and used to assay drug resistance.

[c] After overnight incubation in Penassay Broth containing CM (2.5 or 5.0 μg/ml), the harvested bacteria were washed once with fresh medium and suspended in the original volume of Penassay Broth. One loopful of the bacterial suspension was inoculated in Penassay Broth. After overnight incubation at 37 C, the culture was used to assay drug resistance.

[d] Numbers in parentheses indicate CM inactivation (micrograms of CM inactivated per milligram of bacteria per hour). The values for all CM^s strains were less than 10.

TABLE 4. *Transduction of resistance to chloramphenicol*[a]

Recipient	Transduction frequency[b]	Inactivation of CM[c]
MS353	−6.2	1,600
S1396	−6.8	1,800

[a] The phage lysates were obtained from appropriate strains. The donor strain was S1337; the multiplicity of infection was 0.5. Transductants were subjected to two successive single-colony isolations on plates containing CM (12.5 μg/ml), and resistance to other drugs was determined. All transductants were resistant to CM.

[b] Expressed as the logarithm of the transduction frequency.

[c] CM inactivation was assayed (as in Table 3) after treatment with CM.

Okamoto and Suzuki (16) reported that R factor-resistant *E. coli* inactivates CM through acetylation. The products of CM acetylation were found to be 3-acetoxychloramphenicol and 1,3-diacetoxychloramphenicol (17). It was also reported that a crude extract prepared from a CM-susceptible (K-10) strain catalyzes the forma-

TABLE 5. *Identification of products of chloramphenicol acetylation*[a]

Compound	R_F in chromatographic system		
	A	B	C
Chloramphenicol	0.25	0.23	0.17
3-Acetoxychloramphenicol		0.63	0.37
1,3-Diacetoxychloramphenicol		0.64	0.77
Products formed by S1337 CM^r			
(a)	0.25	0.24	0.18
(b)	0.29	0.44	0.28
(c)	0.46	0.65	0.38
Products formed by S1477 CM^r			
(a)	0.25	0.20	0.17
(b)	0.29	0.43	0.29
(c)	0.45	0.63	0.39

[a] Ascending chromatography with thin-layer sheets and solvents as follows: A, silica gel in benzene-*n*-butanol (90:10); B, silica gel in *n*-butanol-chloroform-acetic acid (10:89.5:0.5); C, silica gel in chloroform-methanol (95:5).

tion of small amounts of a product of CM acetylation having the chromatographic behavior of 3-acetoxychloramphenicol (17). Recently, it was found that CM-resistant strains of *E. coli*, *P. mirabilis*, and the *Klebsiella-Aerobacter* group isolated from clinical sources and lacking R factors transferable by conjugation also inactivate CM through acetylation (Mitsukashi et al., *submitted for publication*).

In naturally occurring strains of staphylococci, it was reported that CM-resistant strains inactivate CM through acetylation (19), suggesting that the product of CM-acetylation may be 3-acetoxychloramphenicol (Okamoto, *unpublished data*).

By using a larger number of naturally occurring strains of staphylococci, it was confirmed that CM-resistant strains inactivate CM, whereas CM-susceptible strains do not. These results strongly suggest that CM resistance in staphylococci derived from clinical sources can be accounted for by inactivation of the drug through acetylation. This is supported by the fact that elimination of CM resistance in staphylococci is accompanied by loss of the capacity to inactivate CM by acetylation.

The genetic determinant for CM resistance in staphylococci has been shown to be transducible with typing phages (2, 3) or with prophages derived from CM-resistant donor strains (Kasuga and Mitsuhashi, *submitted for publication*). These CM-resistant transductants acquire the ability to inactivate CM, indicating that CM resistance in naturally occurring strains of staphylococci is attributable mainly to the capacity to inactivate the drug through acetylation.

Inducible resistance to macrolide antibiotics in staphylococci has been reported (6, 20). It was noted that erythromycin is an inducer in spite of its inhibition of protein synthesis, although the mode of inducible resistance to macrolides has not been investigated fully. Similarly, it was found that CM acts as an inducer of CM resistance despite being an inhibitor of protein synthesis. The failure of CM to induce resistance at higher concentrations may be attributed to its mode of action as an inhibitor of protein synthesis. The mechanism of induction by CM and inducible resistance in staphylococci will be described elsewhere.

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