

Basis of Chloramphenicol Resistance in Naturally Isolated Resistant Staphylococci

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S. Miyamura (J. Pharm. Sci. 53:604, 1964) noted inactivation of chloramphenicol in cultures of chloramphenicol-resistant *Staphylococcus aureus*. Our recent findings (S. Okamoto and Y. Suzuki, Nature 208:1301, 1965) on drug-inactivating enzymes of *Escherichia coli* carrying R factor (*E. coli* R⁺) prompted this study.

All strains were isolated from patients and supplied by S. Mitsuhashi. They are: *S. aureus* S 1174 CM^rSM^rTC^r, S 1047 CM^rSM^rTC^r, and S 2126 CM^rSM^rTC^r. [S 1174 is resistant to chloramphenicol (CM) and streptomycin (SM), and sensitive to tetracycline (TC). S 1047 is resistant to CM and TC, and sensitive to SM. S 2126 is sensitive to CM, SM, and TC.] Cells were grown in L broth and washed twice with tris(hydroxymethyl)aminomethane (Tris)-Mg-K buffer (S. Okamoto and Y. Suzuki, Nature 208:1301, 1965). The main difficulty was the disruption of staphylococcal cells. They were broken by repeated grindings plus freezings and thawings with silica sand and powdered Dry Ice, extracted by use of buffer, treated with deoxyribonuclease (5 µg/ml), and centrifuged at 10,000 × *g* for 15 min. The supernatant fraction was dialyzed over night against the buffer, and was used as the crude extract.

Inactivation of chloramphenicol was initially tested by the bioassay method as described in the footnotes to Table 1. A benzene extraction method (see below) was used later.

Chloramphenicol was inactivated by crude extracts of S 1174 (Table 1) and S 1047 (data not shown) in the presence of acetyl coenzyme A at rates of from 4 to 60 µg of chloramphenicol per min per mg of protein, depending on the preparation. Cells grown in the presence of chloramphenicol gave a more active preparation than those grown in its absence. Whether this is due to enzyme induction or to selection of the resistant population remains to be tested. The extract of a chloramphenicol-sensitive strain S 2126 failed to inactivate the drug (Table 1). When acetyl co-

enzyme A (CoA) was replaced by adenosine triphosphate (ATP) and CoA, for maximal inactivation it was necessary to add an extract of *E. coli* R⁻ (the 100,000 × *g* supernatant fraction of the

TABLE 1. Inactivation of chloramphenicol by the extracts of *Staphylococcus aureus*^a

Extract source	Rate of chloramphenicol disappearance (µg per min per mg of protein)			
	S2126 CM ^s	S1174 CM ^r		
		Expt 1 ^b	Expt 2	Expt 3 ^c
Extract only.....	—	0	0	<0.01
Extract, ATP, CoA.....	0	1.5	0.4	0.6
Extract, <i>Escherichia coli</i> extract.....	—		0	0
Extract, <i>E. coli</i> extract, ATP, CoA.....	0		3	56
Extract, acetyl CoA.....	0		4	66

^a The reaction mixture contained: 0.1 M Tris-HCl buffer (pH 7.8), 0.06 M KCl, 0.01 M magnesium acetate, 100 µg of C¹²- and C¹⁴-chloramphenicol (30,000 counts per min), crude extract of *S. aureus* (0.5 mg of protein), with or without 2 µmoles of ATP plus 0.02 µmole of CoA, 0.5 µmole of acetyl CoA, or 0.5 mg of *E. coli* R⁻ protein, in a total volume of 0.5 ml. At intervals, 0.1-ml samples were pipetted into 0.4 ml of water at 95 C, cooled after 5 min, and extracted three times with benzene. The rate of chloramphenicol inactivation was estimated from the benzene extractable radioactivities.

^b The experiment was performed by the bioassay method. The reaction mixture was similar to that described above except that *S. aureus* extract (1.2 mg of protein), 30 µg of chloramphenicol (no C¹⁴-chloramphenicol), and 5 µmoles of ATP were used. At intervals, a 0.1-ml portion was pipetted into 5 ml of peptone-glucose medium at 95 C. After 5 min, the medium was cooled and inoculated with 10⁷ cells of *E. coli*; the bioassay of the residual chloramphenicol was performed with suitable controls.

^c Cells were grown in the presence of 5 µg/ml of chloramphenicol.

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French pressure cell extract in Tris-Mg-K buffer). [The *E. coli* R⁻ strain in this study is *E. coli* K-12 CS-2 (P. D. Skaar and A. Garen, Proc. Natl. Acad. Sci. U.S. 42:619, 1956).] This extract causes no inactivation by itself, but apparently serves as the source of acetyl CoA synthetase, in which the staphylococcal extracts were deficient, probably because of the difficulty of cell disruption.

Precipitation of the crude extract of S 1147 or S 1047 with ammonium sulfate yielded chloramphenicol-inactivating activity in the fraction between 30 to 65% saturation. The crude extract lost over half of its activity after 5 min at 60 C, but 10% was still retained after 5 min at 80 C. Activity was completely lost after 5 min at 90 C.

The products of the inactivation reaction with C¹⁴-chloramphenicol were fractionated by paper chromatography with a benzene-methanol-water system (upper phase of the 98:2:2 mixture). Two radioactive peaks were noted, one at the origin and one at an R_F of 0.7. The former is chloramphenicol and the latter is most likely a monoacetylated derivative of chloramphenicol, as judged by the behavior of the authentic monoacetyl

chloramphenicol (Y. Suzuki and S. Okamoto, *in preparation*). No second peak was observed with S 2126 extracts. By measuring benzene-extractable radioactivities of the reaction mixture, the degree of inactivation can also be assessed.

The above findings strongly suggest that only the extracts of chloramphenicol-resistant strains contain an enzyme(s) which inactivates chloramphenicol through acetylation of the drug molecule. This is most likely the mechanism of the drug resistance of those strains.

Though the number of strains investigated was limited, it appears likely, in view of Miyamura's results, that chloramphenicol resistance in staphylococci isolated from patients is usually caused by the same mechanism as reported here. On the other hand, the isolation in the laboratory of chloramphenicol-resistant staphylococci of this type may be impossible, as was the case in the penicillinase-producing staphylococci or *E. coli* R⁺. Further study on these points is presently in progress.

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