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Acquired and Native Resistance of *Staphylococcus aureus* to Cephalexin and Other β-Lactam Antibiotics[1]

FRITZ H. KAYSER,[2] E. JACK BENNER, AND PAUL D. HOEPRICH

Section of Infectious and Immunologic Diseases, Department of Internal Medicine, School of Medicine, University of California, Davis, California 95616

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Staphylococcus aureus cells that are initially susceptible to cephalexin can be induced to acquire intrinsic resistance to cephalexin in comparatively few steps. Concomitantly, resistance to cephalothin, oxacillin, and dicloxacillin increases. By population analysis, there is heteroresistance to cephalexin in some strains of *S. aureus*. Heterogeneity in colonial morphology on prolonged incubation in the presence of subinhibitory concentrations of cephalexin may constitute an expression of such heteroresistance.

Some strains of *Staphylococcus aureus* are resistant to the penicillins and cephalosporins by mechanisms that are independent of β-lactamase inactivation of these antibiotics; such strains are of increasing clinical importance (1). Intrinsic resistance may be acquired by a multistep increase in resistance in the course of serial transfer on culture media containing progressively greater concentrations of a penicillin (3). In addition, intrinsic resistance can be a property possessed by some strains never known to have been exposed to a penicillin or a cephalosporin. Such native intrinsic resistance is often termed "methicillin" resistance (7, 9).

In earlier work (5), we reported that cephalexin, the newest of the semisynthetic cephalosporins, was active at therapeutic concentrations against only 6 of 70 strains of *S. aureus* with intrinsic "methicillin" resistance. Since cephalexin is less active against *S. aureus* than other β-lactam antibiotics (4, 5, 12), it seemed reasonable to suppose that initially susceptible strains might acquire resistance to it in a multistep pattern. In this report, we document the step-wise development in vitro of acquired intrinsic resistance by *S. aureus* to cephalexin, concomitant with cross-resistance to other cephalosporins and penicillins.

MATERIALS AND METHODS

S. aureus. Seven of the eight strains of *S. aureus* that were studied were recent clinical isolates. The special properties of these strains are given in Table 1.

Culture medium. Commercial formulations of Brain Heart Infusion (Bioquest Laboratories, Baltimore, Md.) as broth and as agar were used.

Antimicrobial agents. Cephalexin and cephalothin were supplied by Eli Lilly & Co.; oxacillin and dicloxacillin were supplied by Bristol Laboratories.

Susceptibility testing. Using a simplified modification of the Steers replicator (11), the minimal inhibitory concentrations (MIC) of original strains and variants that were developed were determined by using an agar-dilution technique. Overnight cultures in broth were diluted in sterile 0.9% NaCl solution to provide inocula of 3×10^5 to 6×10^5 bacteria at each point on the agar plate. The test plates were examined for growth after 24 hr and again after 48 hr of incubation at 37 C. In some experiments, testing was done with inocula of 10 to 50 viable units per ml. The susceptibility of several single-colony isolates was examined by using the replica plate technique of Lederberg and Lederberg (10).

Population analyses. Disaggregation of 24-hr broth cultures (by brief, controlled exposure to 20-kc sound) was followed by serial dilution (0.9% NaCl) and surface inoculation of agar petri plates containing 0 to 512 μg of either cephalexin, cephalothin, oxacillin, or dicloxacillin per ml. Colony counts after 48 and after 72 hr of incubation at 37 C allowed calculation of the per cent of viable units resistant to each concentration of antimicrobial agent. A single colony from the plate containing the highest concentration of cephalexin at which there was growth was picked to inoculate a tube of broth; population analysis was then repeated as described.

Development of resistant strains. Development of resistance was attempted by both a disc method (8) and by serial transfer in broth. Single colonies growing within the zone of inhibition about a disc containing 30 μg of cephalexin were subcultured in broth prep-

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[2] Visiting Microbiologist for the year 1969 from the Institute of Medical Microbiology, University of Zurich, Zurich, Switzerland.

TABLE 1. *Eight strains of S. aureus were used for study of acquisition of intrinsic resistance to cephalexin and other β-lactam antibiotics*

Isolate	Susceptible to		Penicillinase production
	Penicillin G	Methicillin	
Pr	−	+	0
Ps	+	+	0
Ps 80[a]	+	−	0
545	−	+	+
611	−	+	+
548	−	+	+
E-208	−	−	+
49	−	−	0

[a] Propagation strain for staphylococcal bacteriophage type 80.

aratory to a second exposure to a cephalexin disc. The procedure was repeated until there was no zone of inhibition around the disc.

Tubes containing serial dilutions of cephalexin were inoculated with approximately 10^7 viable units per tube from an overnight broth culture of the strain of *S. aureus* under study. After 24 hr at 37 C, samples containing approximately 10^7 viable units were taken from the tube with the highest concentration of cephalexin in which growth was visible to inoculate a new set of tubes containing cephalexin.

RESULTS

Strains 49 and E-208 (Fig. 1) were comprised of staphylococci with differing levels of resistance to cephalexin; i.e., they were heteroresistant to cephalexin. Heteroresistance was also present with oxacillin. The basic resistance (that concentration of an antimicrobial agent that can be tolerated by 20% of the cells of the bacterial strain under study) to cephalexin was higher than it was to oxacillin; however, the maximum resistance of a minority population of each strain was similar with cephalexin and oxacillin.

With the disc method, acquired intrinsic resistance was successfully induced in three strains (Table 2). Only three single-colony isolations of strain 545 were necessary to yield a variant exhibiting complete resistance by the disc test. The MIC of cephalexin increased eightfold from parent to third-step variant. With strain 611, eight exposures to cephalexin were required to obtain a variant that was not inhibited by the 30-μg disc. With the Pr strain, 11 exposures were necessary. According to replica plate MIC testing, there was simultaneous increase in resistance to cephalothin, oxacillin, and dicloxacillin. Strain Ps failed to acquire intrinsic resistance by the disc-agar diffusion method.

Serial transfer in broth (Table 3) yielded a

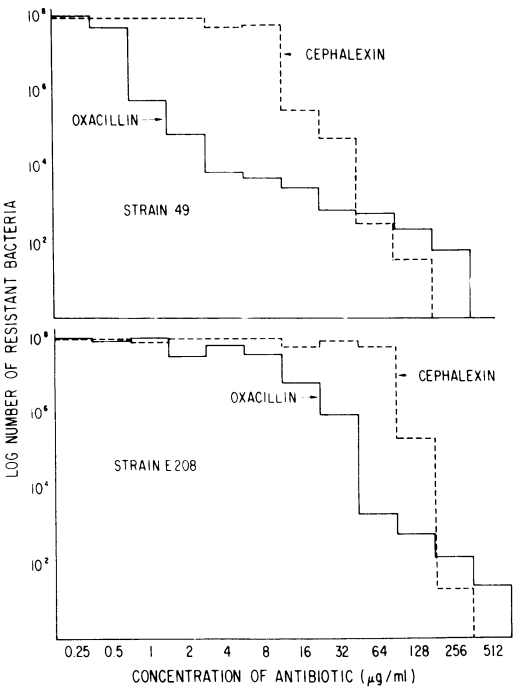

FIG. 1. *Population analysis of the susceptibility of S. aureus, strains 49 and E-208, to cephalexin and oxacillin. Although both strains were heteroresistant, the basic resistance to cephalexin exceeded that of oxacillin with both strains.*

TABLE 2. *Induction of intrinsic resistance in three strains of S. aureus by use of discs containing 30 μg of cephalexin in an agar-diffusion system*[a]

Strain/passage	Minimal inhibitory concn (μg per ml)			
	Cephalexin	Cephalothin	Oxacillin	Dicloxacillin
545/0	8	0.5	0.5	0.25
545/3	128	4	4	2
611/0	4	0.25	0.25	0.125
611/8	256	8	8	4
Pr/0	4	0.25	0.25	0.25
Pr/11	64	1	2	1

[a] There was a concomitant increase in resistance to cephalothin, oxacillin, and dicloxacillin.

markedly resistant variant from strain Ps after 12 transfers. Eight, seven, and five transfers were necessary with strains 545, 611, and Ps, respectively. Again, there was concomitant acquisition of intrinsic resistance to cephalothin, oxacillin, and dicloxacillin.

Resistant variants were disclosed in the course of population analyses. In Fig. 2, the number of

survivors is plotted against the concentration of cephalexin for parent strains Ps 80 and 545 and three strains developed from each parent strain. Only three to four selections were necessary with both strains to obtain highly resistant variants.

To obtain more accuracy in population studies and to eliminate the possible influence of penicillinase on the results, strain Ps was used for further analyses. In Fig. 3, the numbers of surviving staphylococci are related to the concentrations of cephalexin for the parent strain Ps and two resistant variants derived from Ps. Arrows mark the concentrations at which the variants were selected. Note that the buildup of resistance accelerated with each selection step. After only three steps, a highly resistant variant was isolated.

A remarkable heterogeneity of colony morphology resulted when sonically disaggregated strain Ps was incubated at 37 C for 48 hr on agar containing 0.5 to 4.0 μg of cephalexin per ml. The colonies differed in size and there was a high degree of irregularity (Fig. 4, 5). The large colonies were often composed of a flat, translucent zone and a denser, yellow part (Fig. 4). Protru-

TABLE 3. *Induction of intrinsic resistance to cephalexin in three strains of S. aureus by serial transfer in broth*[a]

Strain/ passage	Minimal inhibitory concn (μg per ml)			
	Cephalexin	Cephalothin	Oxacillin	Dicloxacillin
545/0	8	0.5	0.5	0.25
545/4	64	2	2	1
545/9	512	32	32	32
611/0	4	0.25	0.25	0.125
611/3	64	2	2	1
611/9	512	16	16	8
Ps/0	2	0.125	0.125	0.125
Ps/4	128	2	2	0.5
Ps/12	512	32	32	32

[a] There was a concomitant increase in resistance to cephalothin,

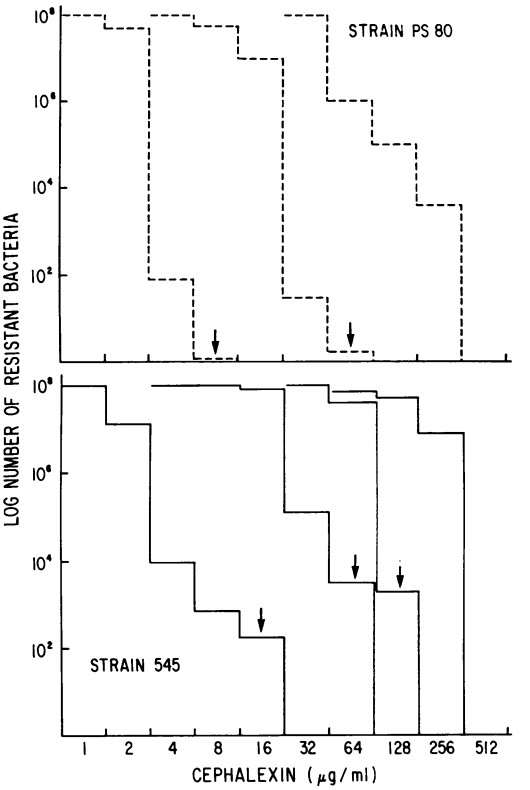

FIG. 2. *Number of surviving S. aureus is presented as a function of the concentration of cephalexin in the culture medium for parent strains Ps 80 and 545 and for three variants from each strain picked from plates containing cephalexin, as designated by the arrows.*

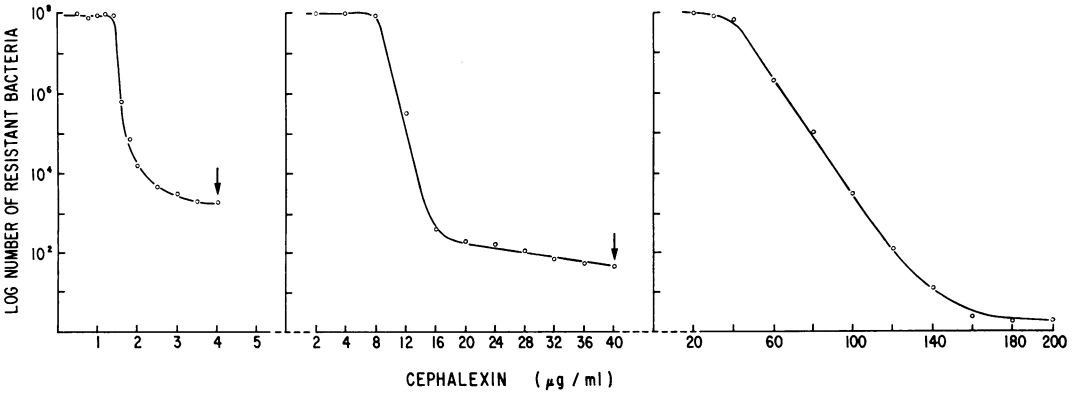

FIG. 3. *Number of resistant S. aureus is related to the concentration of cephalexin for parent strain Ps and two variants derived at the concentrations marked by the arrows.*

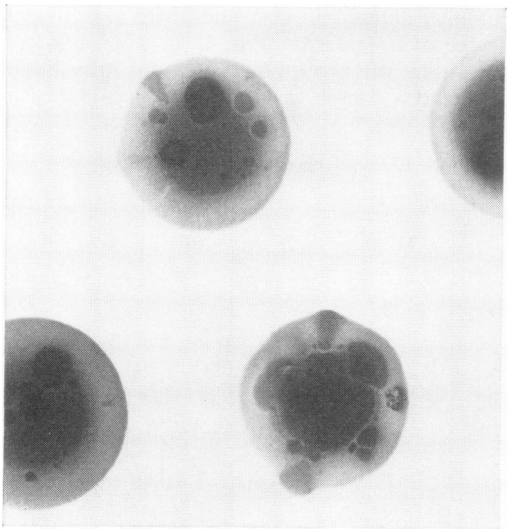

FIG. 4. *Heterogeneity in colony morphology of S. aureus strain Ps after 48 hr of incubation in the presence of 1.2 μg of cephalexin per ml. × 100.*

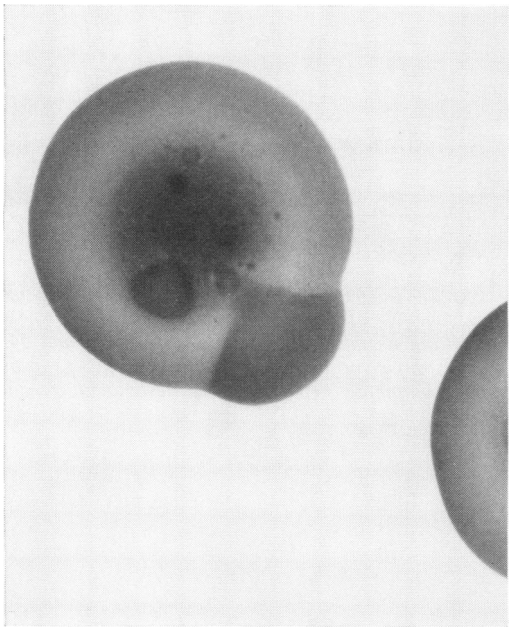

FIG. 5. *Protuberant growth distorting the circumference of a large colony of S. aureus strain Ps after 48 hr of incubation in the presence of 1.5 μg of cephalexin per ml. × 100.*

sions frequently marred the circumference of the large colonies (Fig. 5). The small colonies often contained daughter colonies that differed in density and color. Similar daughter colonies were also observed within the large colonies. These

TABLE 4. *Marked heterogeneity in colonial morphology after 48 hr of incubation of strain Ps in the presence of subinhibitory concentrations of cephalexin*[a]

Colonies		Minimal inhibitory concn (μg per ml)	
Source	No. examined	Mean	Standard deviation
No cephalexin.....	55	2 ± 0	0
Large colonies, 2.5 to 4 μg of cephalexin per ml....	20	5.47 ± 0.11	0.50
Small colonies, 2.5 to 4 μg of cephalexin per ml....	20	3.96 ± 0.20	0.92
Cephalexin (0.5 to 2 μg per ml)			
Parent colony.....	25	2.17 ± 0.07	0.38
Protuberance.....	20	6.68 ± 0.68	3.06
Cephalexin (0.5 to 2 μg per ml)			
Parent colony.....	15	2 ± 0	0
Intracolony daughter.....	15	5.46 ± 0.45	1.76

[a] Variation in susceptibility of cephalexin accompanied the alteration in colonial morphology.

peculiarities of colonial morphology were not evident after 12 hr of incubation.

Differences in susceptibility to cephalexin were found when various portions of the irregular colonies were compared with colonies grown on agar free from cephalexin (Table 4). For testing of susceptibility, portions of colonies selected according to morphology were inoculated into broth, incubated overnight, and then diluted to provide inocula of 10 to 50 viable units. Fifty-five colonies from antibiotic-free agar exhibited uniform susceptibility to 2.0 μg of cephalexin per ml. In addition, with the replica plate method (10), in which the inoculum is higher, 771 of 812 colonies yielded cells that were inhibited by 2.0 μg of cephalexin per ml; 41 were inhibited by 2.5 μg per ml. Regular, large colonies were more resistant than small colonies. Intracolony daughter colonies and protuberances were more resistant to cephalexin than the remainder of the colony. Similar, though less striking, results were obtained with first and second step variants.

DISCUSSION

In strains of *S. aureus* with native intrinsic resistance, at the same time the basic resistance to oxacillin was low, the basic resistance to cephalexin was high. For example, the basic resistance

of strain 49 was 16 times higher for cephalexin than for oxacillin. These data not only substantiate the earlier observations of the comparatively low potency of cephalexin (4, 5, 12), but also define a characteristic profile of activity in relating degree of resistance to fraction of staphylococcal population.

Acquisition of intrinsic resistance to cephalexin was readily achieved. The buildup of resistance followed a multistep pattern. However, unlike other β-lactam antibiotics, the steps were huge since as few as three yielded variants resistant to high concentrations of cephalexin.

It is possible that rapid, multistep acquisition of intrinsic resistance can be achieved during treatment of staphylococcal infections with cephalexin. However, should such variants arise, it may be that they would also be reduced in virulence after the fashion of variants with acquired intrinsic resistance to oxacillin (7).

Unquestionably, increased resistance to cephalexin is associated with increased resistance to cephalothin, oxacillin, and dicloxacillin. Yet, it cannot be said that complete cross-resistance exists; the term decreased susceptibility is preferable. It is possible that a stepwise increase in resistance results from independent mutations at genetic loci that direct the synthesis of target sites of the β-lactam antibiotics, affecting, with each step, a reduction in the affinity of antibiotic: target site. The isolation of variants with different degrees of resistance could be explained in this way, since mutations at different loci might result in different degrees of resistance. Only a genetic analysis can confirm or refute this hypothesis. Since cephalexin is a β-lactam antibiotic of low potency, it may be of particular utility to the study of the multistep pattern of development of resistance.

Colony size correlates with the observed differences in resistance. Thus, the more resistant cells are inhibited less and form large colonies; the less resistant cells are restrained in growth and form small colonies.

Heterogeneity in colonial morphology may reflect mutations occurring during growth, since young colonies were morphologically homogeneous. If, as colonies age, mutants with different degrees of resistance arise, their multiplication at the edge of a colony would result in a protuberance or an intracolony daughter if centrally located.

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