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Effect of the Source of Mueller-Hinton Agar and Resistance Frequency on the Detection of Methicillin-Resistant *Staphylococcus aureus*

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Inconsistencies in the results of disk diffusion tests of oxacillin against *Staphylococcus aureus* that occurred when using commercially prepared Mueller-Hinton agar from different sources led us to evaluate the ability of media from different sources to detect resistance to oxacillin, methicillin, and nafcillin in *S. aureus*. Mueller-Hinton agar from five manufacturers was prepared in our laboratory and used for standard disk diffusion and agar dilution tests. Ten oxacillin-resistant *S. aureus* isolates, of which three were definitive-resistant and seven were occult-resistant, were examined. All definitive-resistant strains were resistant to all three antimicrobial agents on four out of five agars. The occult-resistant strains were consistently detected as resistant on only one of the agars. With only slight differences, oxacillin, methicillin, and nafcillin resistance was more readily detected by disk diffusion and agar dilution when initially incubated at 30°C, and extended incubation improved the detection. The frequency of resistance within a population of occult-resistant cells was low compared with the frequency within a population of definitively resistant cells. The heterogeneity of colony morphology and apparent growth rates within a population of occult-resistant cells contributed to the problem of detecting some resistant isolates. Definitive-resistant isolates were characterized by a very high and stable frequency of resistance. Occult-resistant strains were characterized by a lower frequency of resistance, although the true frequency of resistance may be difficult to ascertain because of heterogeneity in growth rates.

Oxacillin (OX), methicillin (ME), and nafcillin (NF) are penicillinase-resistant penicillins used in the treatment of serious *Staphylococcus aureus* infections. It is important to identify clinical isolates that are resistant to these antimicrobial agents for treatment and epidemiological purposes.

The detection of *S. aureus* strains that are resistant to penicillinase-resistant penicillins, also generally known as ME-resistant *S. aureus*, has been a continuing problem for clinical microbiologists since the first reports of resistance to these drugs in 1961 (1, 13). Recommendations for improving the detection of these isolates include (i) incubation of test media at 30 or 35°C instead of 37°C (2, 5, 7, 18, 21), (ii) extension of the incubation time (2, 5), (iii) increasing the inoculum size (5), and (iv) addition of sodium chloride or other osmotic stabilizers to the test medium (2, 5, 18, 22).

Automated susceptibility test systems (6, 8, 9) and standard microdilution test systems (2, 11) have been criticized as unreliable for the detection of OX, ME, and NF resistance in *S. aureus*. However, Thornsberry and McDougal (22) have recently described a reliable and practical microdilution method for detecting resistance to penicillinase-resistant penicillins in *S. aureus*. For their method they recommend that Mueller-Hinton broth (MHB) be supplemented with cations and 2% sodium chloride. This recommendation is consistent with previous reports which suggest that the osmolality of the growth medium influences the test results.

The Kirby-Bauer (3) disk diffusion test has been generally regarded as reliable for the detection of these resistant isolates when performed at 35°C (2, 7, 10, 21). However, increasing reports of discrepancies and interpretation problems led Thornsberry and McDougal (14) to propose that the disk content and zone diameter interpretive breakpoints for all three antimicrobial agents be changed to correlate better with MIC breakpoints as determined by using cation-supplemented MHB with 2% NaCl. They did not consider whether the source of Mueller-Hinton agar (MHA) had an effect on the accuracy and reproducibility of the disk diffusion susceptibility results of either the established or the proposed protocol.

Our experience and the experience of others (P. M. Terry, D. R. Lonsway, and J. E. McGowan, Jr., Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, C330, p. 366) is that at least two types of OX-, ME-, and NF-resistant *S. aureus* clinical isolates are commonly seen in routine disk diffusion susceptibility tests. The first, or definitive-resistant (DR) type, appears as confluent growth up to the edge of the OX, ME, and NF disks, which leads to an unambiguous interpretation of the test result. The second, or occult-resistant (OR) type, has a more subtle resistance and is evidenced by a haze of growth around the disks which is often recognized only when the plate is examined with transmitted light. The term occult-resistant emphasizes the hidden nature of the resistance in routine susceptibility tests.

Here we present evidence that the source of MHA is a primary factor in the ability to detect OX-, ME-, and NF-resistant *S. aureus* strains by disk diffusion and agar dilution tests. We present additional evidence based upon the frequency of resistance which explains why OR isolates are more difficult to detect than DR isolates. We have primarily focused on OX resistance for the in-depth analyses, since we believe, as do others (22), that in most instances resistance to penicillinase-resistant penicillins is

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more readily detected with OX. In addition, OX is routinely used and tested in our institution.

MATERIALS AND METHODS

Bacteria. Three DR isolates (DR1 to DR3) were obtained from the clinical microbiology laboratory of the Wadsworth Veterans Administration Hospital, three OR isolates (OR4 to OR6) were obtained from the clinical microbiology laboratory of the San Francisco General Hospital, and an additional four OR isolates (OR7 to OR10) were obtained from the clinical microbiology laboratory at the University of California at Los Angeles Medical Center. All isolates produced beta-lactamase, were resistant to erythromycin and clindamycin, were susceptible to vancomycin, and gave OX MICs of >16.0 μg/ml when tested by a standard microdilution method with cation-supplemented MHB containing 2% NaCl (22). The three DR isolates and one of the seven OR isolates (OR9) were gentamicin resistant, and the remaining OR isolates were gentamicin susceptible. Quality control strains for routine susceptibility tests included *S. aureus* ATCC 25923 and ATCC 29213. All clinical isolates and ATCC quality control strains were stored in *Brucella* broth with 15% (vol/vol) glycerol at −70°C.

Media. Dehydrated MHA was kindly supplied by the following manufacturers and designated as noted: A, lot 8304-117, Acumedia Manufacturers, Inc., Baltimore, Md.; B, lot C6DNFY, BBL Microbiology Systems, Cockeysville, Md.; C, lot DM3428, Scott Laboratories, Inc., Fiskeville, R.I.; D, lot 709908, Difco Laboratories, Detroit, Mich.; and E, lot 380170, GIBCO Laboratories, Madison, Wis. The media were prepared simultaneously by following the instructions of the manufacturers. The National Committee for Clinical Laboratory Standards protocols M2-T3 (15) and M7-T (16) were followed for preparation of disk diffusion plates and agar dilution plates, respectively, the latter containing OX and NF at concentrations of 0.25 to 16.0 μg/ml and ME at concentrations of 0.5 to 32.0 μg/ml.

Antimicrobial agents. Antimicrobial reference powders were kindly provided by the following manufacturers: OX and ME by Bristol Laboratories, Syracuse, N.Y., and NF by Wyeth Laboratories, Philadelphia, Pa.

Antimicrobial disks. OX (1-μg) and ME (5-μg) disks were obtained from General Diagnostics, Warner-Lambert Co., Morris Plains, N.J., Difco, and BBL. NF (1-μg) disks were obtained from General Diagnostics and BBL; they were not available from Difco.

Disk diffusion and agar dilution MIC tests. All disk diffusion and agar dilution MIC tests were performed in duplicate as recommended by the National Committee for Clinical Laboratory Standards protocols M2-T3 and M7-T, with the following exceptions: (i) inocula were prepared by suspending fresh cell paste in MHB and adjusting to a turbidity equivalent to a McFarland 0.5 standard, and one source of inoculum was used to compare the five different sources of media; (ii) disk diffusion tests were incubated for 16 to 18 h at 35°C and for an additional 24 h at 30°C; (iii) two identical plates of media from each manufacturer were inoculated for agar dilution studies, one plate being incubated for 16 to 18 h at 35°C and then for an additional 24 h at 30°C, and the other plate being incubated at 30°C for the entire period; (iv) disk diffusion tests for OX, ME, and NF were examined under transmitted light; and (v) in the agar dilution test, the MIC was defined as the lowest concentration of OX, ME, or NF which showed no growth, and the plates were examined by being viewed against a black nonreflecting surface under reflected light.

Disk diffusion interpretive breakpoints. The susceptible, intermediate, and resistant breakpoints were: OX and NF, ≥13, 11 to 12, and ≤10 mm; and ME, ≥14, 10 to 13, and ≤9 mm, respectively.

Agar dilution interpretive breakpoints. Isolates with MICs of >2.0 μg/ml for OX and NF and >8.0 μg/ml for ME were considered resistant to the respective drug.

Frequency of resistance. The frequency of OX resistance for a particular isolate was measured by plating dilutions of a suspension of cells adjusted to a McFarland no. 1 standard (prepared by suspending fresh colonies isolated on sheep blood agar plates into saline) onto Difco MHA plates with and without OX (2 μg/ml). All CFU, regardless of morphology and pigment, that grew on the media containing OX after 48 h of incubation at 35°C were counted as resistant. A kinetic analysis of the frequency of OX resistance was performed by using one OR isolate (OR7), one DR isolate (DR2), and two sources of MHA (B and C). OX-resistant isolates of the OR and DR types were readily detected on medium B and poorly detected on medium C. The frequency of resistance was measured after incubation at (i) 35°C for 24, 48, and 72 h; (ii) 35°C for 24 h and then 30°C for the next 24 (48 h) and 48 h (72 h); and (iii) 30°C for 24, 48, and 72 h.

Coagulase. Slide and tube coagulase activity was detected by using citrated rabbit plasma. Tube coagulase activity was checked after 4 and 24 h of incubation at 35°C.

RESULTS

Disk diffusion testing. Media from all five sources gave similar and satisfactory results with *S. aureus* quality control strain ATCC 25923 for disk diffusion tests. However, for the three DR and seven OR strains described here, there were marked differences in the susceptibility results obtained with the different media. All three DR isolates showed definitive resistance to OX (Fig. 1A), ME, and NF (data not shown) on all media except medium C. On medium C, two isolates were OX resistant and one isolate was OX intermediate; however, all three isolates were either susceptible or intermediate to ME and NF after 16 to 18 h of incubation at 35°C. Only one of these isolates was detected as resistant to ME and NF after an additional 24 h of incubation at 30°C. The other two isolates remained susceptible or intermediate, and the OX-intermediate isolate was resistant after the additional 24 h of incubation at 30°C.

OR isolates exhibited a haze of growth around the OX disk (Fig. 1B) and the NF and ME disks (data not shown). With transmitted light, the OR isolates were distinct from susceptible isolates (Fig. 1C). Table 1 shows the number of OR strains (from a total of seven) that each of the five media failed to detect as resistant to the three antimicrobial agents, whether incubated for 16 to 18 h at 35°C or 16 to 18 h at 35°C followed by an additional 24 h at 30°C.

Disks from three manufacturers were used in this study, and there were no significant differences between them, except that one DR isolate demonstrated a "target" of growth with the ME disk from one source on medium C.

Agar dilution testing. Media from all five sources gave similar and satisfactory results with *S. aureus* quality control strain ATCC 29213 in agar dilution tests. However, significant differences in the susceptibility results were seen when the DR and OR isolates were tested on the various media. All DR isolates were resistant to all three drugs on all media except medium C. With medium C, only two of the three DR isolates were resistant to all three antimicrobial agents, and the third strain was resistant to OX but susceptible to ME and NF after either an initial 16 to 18 h of incubation at 35°C

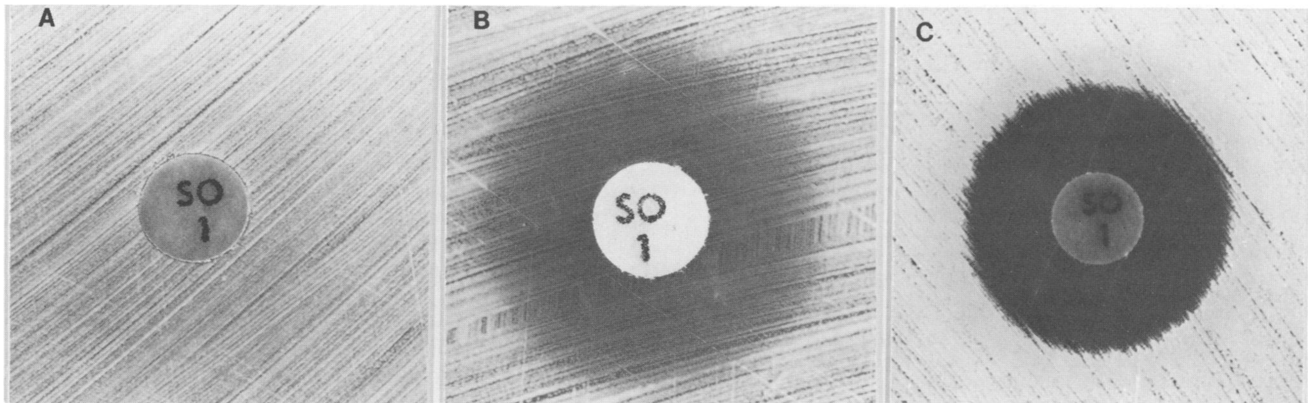

FIG. 1. OX (1 μg) disk diffusion testing of (A) a DR isolate, (B) an OR isolate, and (C) a susceptible isolate. Note appearance of growth around the disks after 18 h of incubation at 35°C.

or an additional 24 h of incubation at 30°C. In contrast, this isolate was resistant to all three antimicrobial agents after just an initial 16 to 18 h of incubation at 30°C.

Although the OR isolates grew luxuriantly on all agar dilution plates without antimicrobial agents, the MICs on media with antimicrobial agents were often difficult to interpret. There was frequently a slight haze of growth or a countable number of colonies of various sizes, but frequently, pinpoint colonies were observed that occasionally decreased in number at higher concentrations of drug. In the interpretation of these results, the distinction between artifact and slight growth (haze) was often subjective.

Table 2 shows the number of OR isolates (from a total of seven) that each of the five media failed to detect as resistant to the three antimicrobial agents under the different temperature and incubation conditions.

Frequency of resistance. The frequency of resistance for the two DR isolates was 100% (Table 3), which was reproduced at least twice. The colonies of DR isolates which grew on MHA with OX (2 μg/ml) were uniform in size, shape, and pigmentation (Fig. 2A). In contrast, the frequency of resistance of the OR isolates ranged from 0.09 to 0.12%. The colonies of OR isolates which grew on MHA with OX (2 μg/ml) were heterogeneous in size, shape, and pigmentation (Fig. 2B). In addition, all OR isolates were slide coagulase (clumping factor) positive, whereas the DR isolates were uniformly slide coagulase negative. Another study (C. J. Hinnebusch and D. A. Bruckner, manuscript in preparation) indicates that a majority of OR isolates were susceptible to gentamicin, whereas DR isolates were usually resistant. OR isolates with the lowest frequencies of resistance for OX were the most difficult to detect by both the disk diffusion and the agar dilution tests.

The differences in colony size within a population of cells from OR isolates suggested differences in growth rates. The frequency of OX resistance within populations of OR and DR isolates was measured as a function of time, temperature, and source of MHA (Table 4). Previous experiments revealed that in disk diffusion and agar dilution susceptibility tests, OX, ME, and NF resistance in OR and DR isolates was readily detected on medium B. Medium C was the only medium on which resistance in all DR isolates was not readily detected. These results were confirmed by the kinetic analysis of OX resistance frequencies (see above). On medium B at 35°C, the frequency of resistance of the DR isolate remained constant over 72 h. At 30°C there was a 24-h lag in observable growth of resistant colonies; however, at 48 h the frequency was the same as at the higher temperature. On medium C, resistance of the DR isolate was not demonstrated at 35°C or when first incubated at 35°C and then at 30°C. There was incomplete (by comparison with medium B) demonstration of resistance when the plates were maintained at 30°C for the entire 72 h. In contrast, the frequency of OX resistance of the OR isolate on medium B

TABLE 1. Disk diffusion testing of seven OR isolates of *S. aureus*

Antimicrobial agent	Incubation conditions[a]	No. of isolates missed on medium[b]:				
		A	B	C	D	E
OX	1	1	0	0	3	3
	2	0	0	0	0	0
ME	1	6	0	7	6	6
	2	1	0	7	0	0
NF	1	2	0	3	3	2
	2	0	0	0	0	1

[a] 1, Incubated at 35°C for 16 to 18 h; 2, incubated at 35°C for 16 to 18 h and reincubated at 30°C for an additional 24 h.

[b] Source of media: A, Acumedia lot 8304-117; B, BBL lot C6DNFY; C, Scott lot DM3428; D, Difco lot 709908; and E, GIBCO lot 380170.

TABLE 2. Agar dilution testing of seven OR isolates of *S. aureus*

Antimicrobial agent	Incubation conditions[a]	No. of isolates missed on medium[b]:				
		A	B	C	D	E
OX	1	1	0	1	4	4
	2	0	0	0	2	3
	3	4	0	1	3	3
	4	2	0	0	3	2
ME	1	4	0	6	6	5
	2	2	0	6	4	1
	3	2	0	4	4	2
	4	0	0	2	0	1
NF	1	2	0	5	5	3
	2	1	0	4	5	1
	3	0	0	4	3	3
	4	0	0	3	3	1

[a] 1, Incubated at 35°C for 16 to 18 h; 2, incubated at 35°C for 16 to 18 h and reincubated at 30°C for an additional 24 h; 3, incubated at 30°C for 16 to 18 h; 4, incubated at 30°C for 16 to 18 h and reincubated at 30°C for another 24 h.

TABLE 3. Characterization of OR and DR isolates of *S. aureus*

Isolate	Source[a]	OX phenotype	Gentamicin phenotype[b]	OX frequency[c]	Coagulase	
					Free	Bound
DR1	WVAH	DR	R	1.0	+	−
DR2	WVAH	DR	R	1.0	+	−
OR6	SFGH	OR	S	8.6×10^{-4}	+	+
OR7	UCLA	OR	S	9.0×10^{-4}	+	+
OR9	UCLA	OR	R	12.0×10^{-4}	+	+

[a] WVAH, Wadsworth Veterans Administration Hospital; SFGH, San Francisco General Hospital; UCLA, University of California at Los Angeles Medical center.

[b] Susceptibility (S) or resistance (R) to gentamicin in a disk diffusion test.

[c] Resistance frequency measured as the total CFU observed on plates containing 2 μg of OX per ml from a suspension of the respective strains diluted to give approximately 200 CFU per plate.

increased with time. As with the DR isolate, the OR isolate grew better at 30°C on medium C; however, the total number of colonies was considerably lower than on medium B.

DISCUSSION

Approximately 75% of clinical bacteriology laboratory workers use the disk diffusion test as their routine susceptibility test method (12). The quality control recommendation described in the current National Committee for Clinical Laboratory Standards disk diffusion protocol M2-T3 (15) is to use quality control strains similar to the more common organisms isolated from clinical specimens; however, no strains that have peculiar in vitro resistance to specific antimicrobial agents are presently available (e.g., OX-resistant *S. aureus*, penicillin-resistant pneumococci, or *Enterobacter* spp. that contain inducible beta-lactamases). Some of these strains are included as part of the College of American Pathology proficiency surveys, but it is unlikely that these strains are routinely used to assess antimicrobial susceptibility test protocols currently in use in clinical laboratories.

S. aureus strains resistant to penicillinase-resistant penicillins are difficult to treat and can quickly become a significant infection control problem if they are not recognized; therefore, it is imperative that clinical laboratory workers be able to quickly and unambiguously detect these strains in clinical specimens.

The evidence presented here clearly indicates that the source of MHA can dramatically affect the results of disk diffusion and agar dilution tests for susceptibility of certain isolates of *S. aureus* to penicillinase-resistant penicillins. Five lots of MHA from five different manufacturers were examined, and there were significant differences in the detection of resistance to OX, ME, and NF when using the different media. Given the undefined nature of MHA, which is composed of peptones, beef infusions, and hydrolysate of casein, starch, and agar, the source of these problems may be extremely difficult to identify. Our results emphasize the need to establish performance criteria for MHA with all types of clinical isolates, including problem organisms such as OX-resistant *S. aureus*. These criteria currently are being defined by a National Committee for Clinical Laboratory Standards subcommittee.

Previous reports indicated that 30°C is a preferred temperature to detect resistance to penicillinase-resistant penicillins; however, our data suggest that there is a relationship between temperature and quality of MHA. Incubation at 30°C may provide better conditions for the detection of resistance when using low-quality media, whereas incubation at 35°C appears to be more desirable when using high-quality media.

We suggest that different lots of MHA, irrespective of source, be quality controlled for the detection of OX-, ME-, or NF-resistant *S. aureus* strains by using an OR *S. aureus* strain. Furthermore, we emphasize the importance of using transmitted light to examine disk diffusion test results for *S. aureus* isolates against OX, ME, and NF. In addition, we suggest that after an initial incubation at 35°C for 16 to 18 h, tests for penicillinase-resistant penicillins and *S. aureus* be re-examined after an additional 24 h of incubation.

ME-resistant *S. aureus* isolates have been described as heteroresistant (20, 22). In our opinion, there is some ambiguity in the literature about the meaning of this term; however, we understand heteroresistance to refer to pheno-

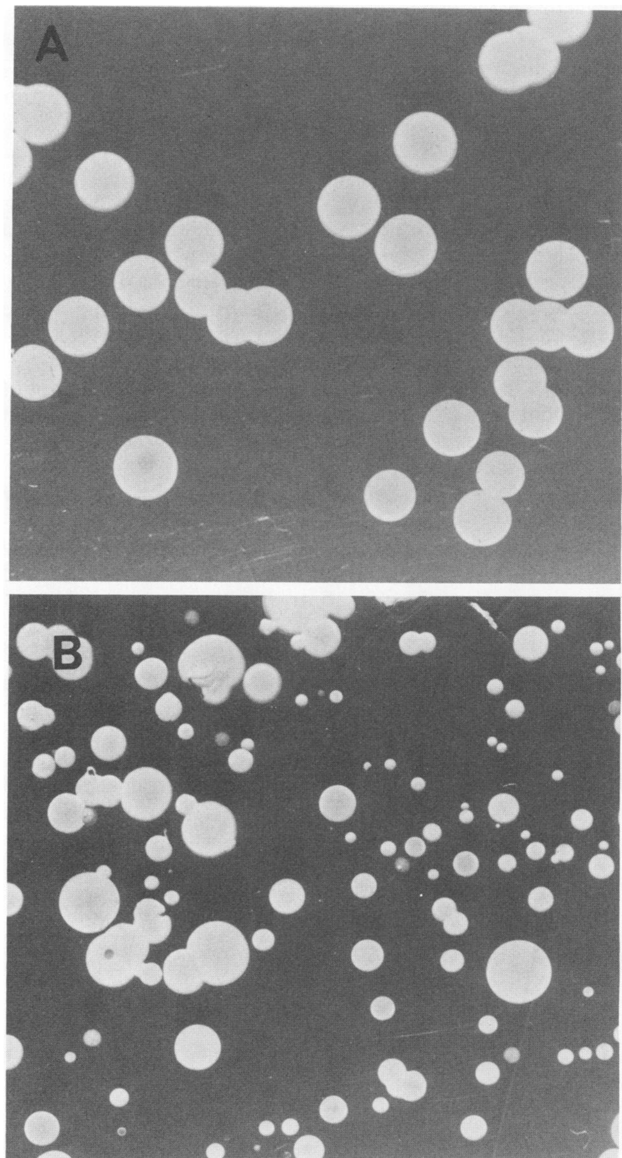

FIG. 2. Colony morphology after 48 h of incubation at 35°C of (A) a DR isolate at a dilution of 10^{-6} and (B) an OR isolate at a dilution of 10^{-3}. Cell suspensions were adjusted to a McFarland no. 1 standard, serially diluted, and plated onto MHA containing 2 μg of OX per ml.

TABLE 4. Growth of DR and OR isolates as a function of time, temperature, and source of MHA

Isolate	MHA source[a]	Incubation conditions[b]	Total CFU/ml (× 10^5) at time (h):		
			24	48	72
DR2	B	1	2,700.0	3,600.0	3,600.0
		2	3,000.0	3,500.0	3,500.0
		3	0	3,200.0	3,600.0
DR2	C	1	0	0	0
		2	0	0	0
		3	0	800.0	1,400.0
OR7	B	1	1.2	12.4	22.9
		2	1.1	5.6	12.4
		3	0	0.9	9.2
OR7	C	1	0	1.5	3.2
		2	0	2.8	4.4
		3	0	4.8	14.2

[a] See Table 1, footnote *b*.
[b] 1, 35°C for 72 h; 2, 35°C for 24 h and then 30°C for the next 48 h; 3, 30°C for 72 h.

typic heterogeneity within a population of cells; i.e., the population contains both ME- (NF- and OX-) susceptible and resistant strains. In this study we have introduced two new terms which we believe better reflect the nature of resistance to these antimicrobial agents in *S. aureus*: definitive-resistant (DR) and occult-resistant (OR) isolates.

DR isolates are characterized by unambiguous agar dilution MIC endpoints or disk diffusion test results. The growth of DR isolates is less influenced by certain environmental factors, such as temperature and length of incubation, and may prove to be less influenced by other factors, such as light, osmolality, chelators, pH, and divalent cations, which have been implicated as important parameters (18, 19). DR isolates grow luxuriantly on media with 2 μg of OX per ml, and the colonies that develop are uniform in size, shape, and pigmentation. The frequency of resistance within a population of DR cells can be as high as 100%. The DR isolates described here may be similar to the homogeneous resistant isolates described by Peacock et al. (17), since those isolates were characterized by a constantly high proportion of resistant cells and clearly defined MIC endpoints.

OR isolates display a hidden type of resistance that is often apparent only after manipulation of culture conditions, especially temperature, length of incubation, and osmolality. Based upon the frequency of resistance measurements, it appears that the OR phenotype is similar to the previously described heteroresistant phenotype. However, evidence presented here and elsewhere (manuscript in preparation) leads us to believe that phenotypic heterogeneity of OR isolates does not reflect susceptible and resistant genotypic heterogeneity. First, a kinetic analysis showed that the frequency of resistance is time dependent and suggests that with prolonged incubation (>72 h) the frequency may approach that of a DR isolate. Second, we showed (manuscript in preparation) that upon repeated cloning of an OR isolate, we were unable to isolate an OX-susceptible strain. If one assumes that the frequency of resistance within a population of OR cells can range from 0.09 to 0.6%, as indicated in Tables 3 and 4, one should have readily isolated OX-susceptible strains.

We believe that OR isolates comprise cells that are genotypically homogeneous for OX resistance; however, in the expression of resistance one observes a phenotypic heterogeneity in growth rates, colony morphology, pigmentation, clumping factor, and various biochemical characteristics. Slow-growing variants or bradytrophs could be either leaky auxotrophic mutants that are susceptible to nutritional deficiencies of various media or mutants influenced by culture conditions or particularly susceptible to inhibitory or lethal factors in the various media.

A recent report (4) indicates that certain ME-resistant *S. aureus* strains contain a novel genetic mapping site which influences ME resistance and could be a transposable genetic element. This report leads to the intriguing, albeit speculative, idea that when OR isolates are grown in the presence of OX, a transposition event is induced which results in the expression of resistance. In addition, the transposition event results in heterogeneous phenotypic expression of a variety of growth and biochemical characteristics. In this regard, it is important to emphasize that phenotypic heterogeneity is only observed when OR isolates are grown in the presence of OX. DR isolates would represent strains in which a past transposition event resulted in a stable integration of the OX resistance determinant(s) and homogeneous expression of growth and biochemical characteristics. We are currently pursuing the possibility that transposition is involved in the phenomenon of OX resistance in *S. aureus*.

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