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Evaluation of Various Antibiotics for Induction of L Forms from *Staphylococcus aureus* Strains Isolated from Bovine Mastitis†

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Forty-five strains of *Staphylococcus aureus* were treated with 11 antibiotics and tested for induction to L forms. Thirty-seven strains were induced to L forms with at least one antibiotic, while eight strains produced no L forms under the conditions used. L forms were induced only with β-lactam antibiotics and with a combination of penicillin and streptomycin. Novobiocin induced no true L forms but induced intermediate forms from seven strains. Strains resistant to penicillin yielded L forms only with β-lactam antibiotics active against penicillin-resistant organisms. Strains failing to yield L forms were susceptible to the antibiotics used, and resistance appeared to play no role in the induction of L forms with these strains.

Staphylococcus aureus is a primary cause of bovine mastitis, often causing a chronic infection that responds poorly to antibiotic therapy (1, 5). The induction of bacterial L forms by compounds that inhibit cell wall synthesis is well described (2, 3, 4). L forms of *S. aureus* have been isolated from the mastitic milk of cows during antibiotic therapy. Sears et al. (9) isolated L forms of *S. aureus* from cows infected experimentally after intramammary treatment with cloxacillin. In a previous study, Owens (8) isolated L forms from cows infected experimentally with *S. aureus* and treated with penicillin. The L-form state has been suggested as a mechanism whereby *S. aureus* can persist in the mammary gland during therapy and reemerge after treatment, with a remanifestation of symptoms (8, 9). *S. aureus* strains isolated from bovine mastitis demonstrated considerable variability for ease of induction to L forms with penicillin (8). β-Lactam antibiotics are used extensively in mastitis therapy, and data on the effects of these and other antibiotics on L-form induction is needed to determine their role in therapy failure.

In this study, strains of *S. aureus* isolated from bovine mastitis were tested for induction to L forms with a variety of antibiotics to determine (i) the ease of induction for different strains and (ii) which antibiotics were most likely to induce L forms.

Forty-five strains of *S. aureus* from our Mastitis Research Laboratory culture collection were evaluated. All were originally isolated from cows with bovine mastitis. Organisms were maintained frozen at −70°C in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) with 20% glycerin. Strains were cultured from frozen stocks on Typticase soy agar (BBL) with 5% bovine blood at 35°C. Brain heart infusion (BHI) agar and broth (BBL) with 5% NaCl, 5% sucrose, 10% horse serum (Sigma Chemical Co., St. Louis, Mo.), and 0.5% yeast extract (Difco Laboratories, Detroit, Mich.) were used for cultivation of L forms. The osmolality of both supplemented BHI media was 1,860 mosmol. *S. aureus* strains were tested for induction to L forms with the following antibiotics: ampicillin, 100 μg/ml; amoxicillin-clavulanic acid (Augmentin), 100 μg/ml; cephalothin, 100 μg/ml; erythromycin, 100 μg/ml; gentamicin, 100 μg/ml; novobiocin, 150 μg/ml; oxacillin, 100 μg/ml; penicillin, 100 U/ml; streptomycin, 100 μg/ml; tetracycline, 100 μg/ml; vancomycin, 100 μg/ml; penicillin-streptomycin, 100 U/ml and 150 μg/ml; and penicillin-novobiocin, 100 U/ml and 150 μg/ml. Concentrations of antibiotics were chosen to approximate concentrations commonly used in intramammary treatment of bovine mastitis. Organisms were suspended in Tryticase soy broth to a turbidity approximating a 0.5 McFarland standard and were incubated for 2 h to ensure that cells were in log phase. To induce L forms, 0.1 ml of the Trypticase soy broth suspension was added to 9.9 ml of the supplemented BHI broth containing the indicated concentration of antibiotic. The BHI broth was prewarmed to 35°C prior to the addition of organisms. Samples were taken after 10 min of incubation and again after 24 h; 0.1 ml of the sample was plated to supplemented BHI agar. Plates were incubated at 35°C and observed daily for 7 days for L-form colonies.

All strains were tested for susceptibility to the various antibiotics by standard methods (7).

Table 1 shows the induction of L forms from the strains

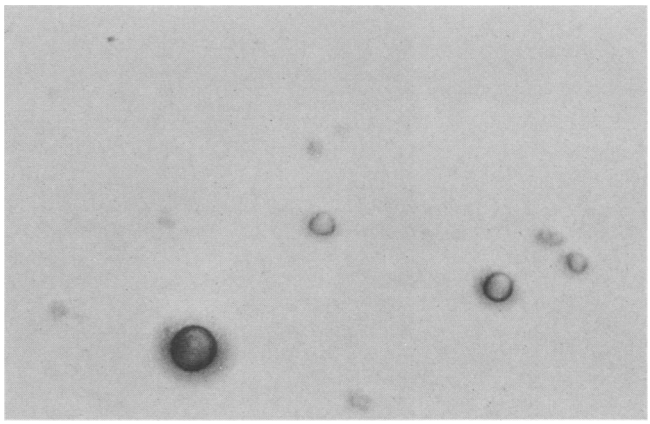

FIG. 1. Typical L-form colonies of *S. aureus* exhibiting classical "fried-egg" colony morphology. Magnification, ×32.

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TABLE 1. Number of *S. aureus* strains induced to L forms after 10 min or 24 h of exposure to selected antibiotics[a]

Antibiotic (concn)	No. of strains producing:							
	L forms				Reverters		Intermediates	
	10 min	24 h	Both	Either	10 min	24 h	10 min	24 h
Ampicillin (100 μg/ml)	18	11	8	22	3	5	14	19
Amoxicillin-clavulanic acid (100 μg/ml)	17	5	3	17	18	3	20	0
Cephalothin (100 μg/ml)	20	16	12	24	2	9	2	6
Erythromycin (100 μg/ml)	0	0	0	0	0	0	0	0
Gentamicin (100 μg/ml)	0	0	0	0	0	0	0	0
Novobiocin (150 μg/ml)	0	0	0	0	0	0	7	0
Oxacillin (100 μg/ml)	21	10	8	22	3	23	19	31
Penicillin (100 U/ml)	20	6	5	21	3	7	8	6
Streptomycin (100 μg/ml)	0	0	0	0	0	0	0	0
Tetracycline (100 μg/ml)	0	0	0	0	0	0	0	0
Vancomycin (100 μg/ml)	0	0	0	0	0	0	0	0
Penicillin-streptomycin (100 U/ml-100 μg/ml)	15	4	3	16	0	0	9	6
Penicillin-novobiocin (100 U/ml-150 μg/ml)	0	0	0	0	0	0	2	0

[a] A total of 45 strains from the Mastitis Research Laboratory culture collection were tested.

tested with the various antibiotics. All β-lactam antibiotics induced L forms from some of the *S. aureus* strains. More L forms were isolated after 10 min of exposure to antibiotic than after 24 h. In addition to typical L-form colonies as represented in Fig. 1, some strains also produced intermediate or reverter colonies. Intermediate colonies (Fig. 2) were typically smaller than parental colonies and lacked the dense core characteristic of true L-form colonies. Subculture of these colonies to bovine blood agar resulted in reversion to the parental form. Reverter colonies exhibited characteristics of both intermediates and L forms (Fig. 3) and displayed several dense core areas with irregular areas resembling parental colonies. Subculture of these colonies to blood agar also resulted in reversion to the parental form.

Table 2 lists antibiotics inducing L forms from the various *S. aureus* strains. Six of the strains were resistant to ampicillin and penicillin by standard test methods. These strains were induced to L forms only by cephalothin, oxacillin, or amoxicillin-clavulanic acid. Seven strains were not induced to L forms under the conditions of this study. All of these strains were susceptible in vitro to the antibiotics used. Novobiocin induced no true L forms but did induce intermediate colonies from seven strains.

The majority of antibiotics used for treatment of bovine mastitis induced L forms from the *S. aureus* strains tested. Novobiocin, streptomycin, and erythromycin did not induce L forms. These antibiotics act by mechanisms other than inhibition of cell wall biosynthesis, thereby reducing the likelihood of L-form induction. The combination of penicillin and streptomycin induced L forms from a number of the strains tested.

Some strains demonstrated a greater tendency toward L-form induction than others. Seven strains yielded no L forms under the conditions used, although all were suscep-

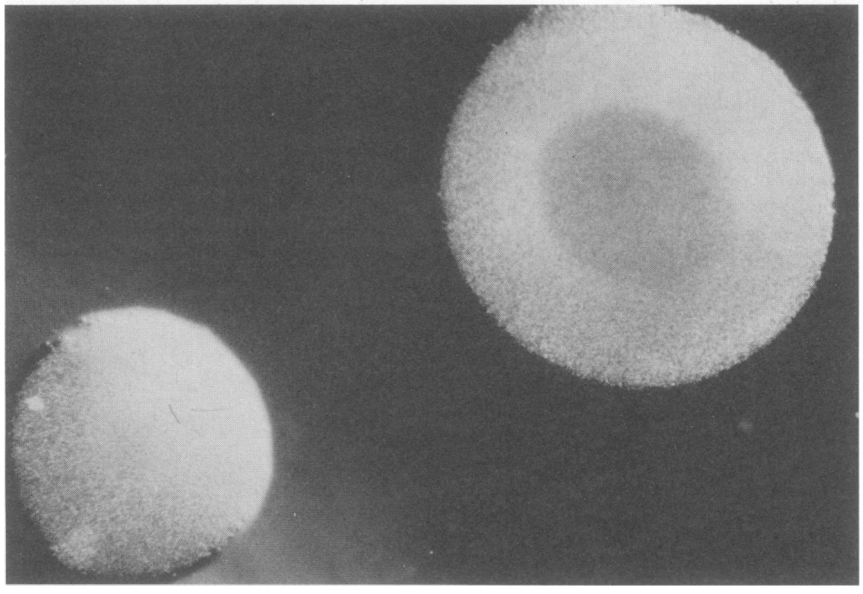

FIG. 2. Intermediate colony of *S. aureus*. Note the lack of a dense core area. Magnification, ×20.

TABLE 2. Antibiotics inducing L forms from selected strains of *S. aureus* of bovine origin

MRL[a] strain no.	Antibiotic(s)[b] inducing L forms	MRL strain no.	Antibiotic(s) inducing L forms
3	None	49	AMC, P, AM
4	None	54[c]	OX
5[c]	OX	57	CL, P-S, AMC, P
6	None	58	AM
8	CL, P, OX, P-S, AMC	62	AMC
12	CL	66	CL, OX, AM
13	CL, P, OX, P-S, AMC, AM	67	CL, AMC, P, AM
16	P-S	71	CL, P, OX, P-S, AMC, AM
17	CL, P, OX, P-S, AMC, AM	74	CL, P, OX, P-S, AMC, AM
18	CL, P, OX, AMC, AM	78	None
19	CL	87	CL, P-S, OX, P, AM
20	None	89	CL, P-S, OX, AMC, AM
21	CL, AM	95	None
22	OX, AM, P, P-S	113	AMC
23	CL, P, OX, P-S, AMC, AM	115	None
24	AMC, AM	118	CL, OX, P, AM
27	CL, P, OX, P-S, AMC, AM	119	P-S
28	AM	470	CL, P, OX, P-S, AMC, AM
30	OX, AM	442[c]	CL
39	CL, P, OX, P-S, AMC, AM	441[c]	CL, OX
41	CL, P, OX, P-S, AMC, AM	443[c]	CL
45	None	440[c]	OX
46	CL, P, OX, P-S, AMC, AM		

[a] Mastitis Research Laboratory.
[b] Abbreviations: OX, oxacillin; CL, cephalothin; P, penicillin; P-S, penicillin-streptomycin; AMC, amoxicillin-clavulanic acid; AM, ampicillin.
[c] Strains resistant to penicillin and ampicillin.

tible to β-lactam antibiotics. Thus, resistance did not play a role in L-form induction. Six strains resistant to penicillin yielded L forms only with β-lactam antibiotics active against β-lactamase-producing *S. aureus*.

The results indicate that some bovine strains of *S. aureus* are induced more readily to L forms. Often within a given dairy herd, a few strains of *S. aureus* predominate, and one strain may be responsible for the majority of infections. If that strain has an increased tendency toward L-form induction, the likelihood of treatment failure would be increased.

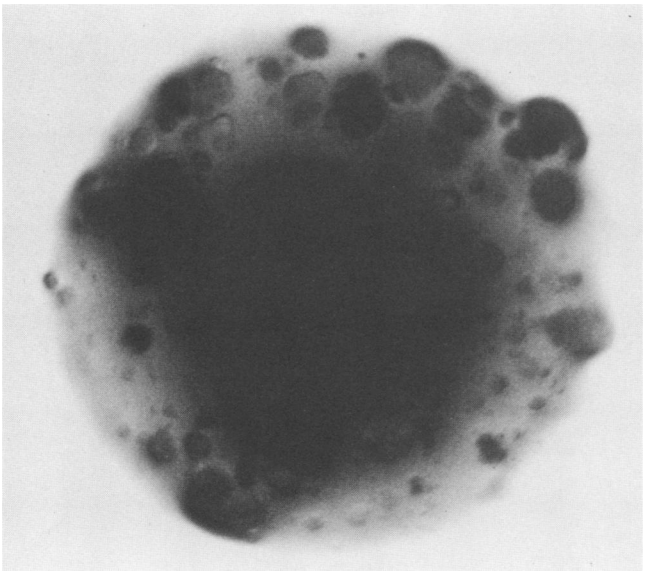

FIG. 3. Reverter colony of *S. aureus* exhibiting multiple core areas. Magnification, ×60.

The formation of unstable L forms, intermediate forms, and reverting forms in vitro suggests this possibility in vivo. Intermediate and reverting forms have been isolated from milk samples of infected cows during therapy (8, 9). Thus, one can envision a possible path from the parent organism to L forms, intermediates, reverters, and back. Green et al. (6) proposed a similar life cycle for L forms of *Enterococcus* (*Streptococcus*) *faecalis*. These workers suggested that under favorable conditions the more stable L forms develop into transitional forms and finally revert completely to parental bacterial forms. Whether this life cycle occurs in vivo has yet to be determined. L-form induction during treatment has been clearly documented. However, the importance of L forms as a mechanism of treatment failure has not been determined. Further work is needed to understand the importance of L forms in bovine mastitis.

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