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NOTES

Methicillin-Resistant Septal Peptidoglycan Synthesis in a Methicillin-Resistant *Staphylococcus aureus* Strain

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In a methicillin-resistant *Staphylococcus aureus* strain, electron micrographs showed that cell wall septa continued to be formed in the presence of methicillin, although they became distorted and enlarged. The results indicated that peripheral cell wall synthesis was inhibited. It is concluded that a methicillin-resistant mode of septal peptidoglycan synthesis is an important determinant of methicillin resistance.

Methicillin resistance is a poorly understood form of resistance to β-lactam antibiotics that is different from that due to drug inactivation through β-lactamase production (5, 6, 14). Recent studies of peptidoglycan syntheses in a methicillin-resistant *Staphylococcus aureus* strain led to the hypothesis (15) that peptidoglycan synthesis involved in thickening of cell walls is methicillin susceptible, whereas septal peptidoglycan synthesis involved in cell division is methicillin resistant. The present study provides further evidence in support of this hypothesis. Electron micrographs showed that cell wall septa, although distorted and enlarged, continued to be formed in the presence of methicillin and indicated that peripheral cell wall synthesis was inhibited. In contrast to a derived methicillin-susceptible strain, lysis did not appear to be a consequence of methicillin treatment of the methicillin-resistant strain. It is concluded that a methicillin-resistant mode of septal peptidoglycan synthesis is an important determinant of methicillin resistance.

Methicillin-resistant *S. aureus* DU4916 and methicillin-susceptible *S. aureus* DU4916S, which was derived from acriflavine-treated strain DU4916 (4), were used in these studies. The responses of growth and peptidoglycan syntheses of these strains to methicillin have previously been studied in detail (15). The methicillin concentrations and sampling times used in the present experiments were selected on the basis of these previous studies (Table 1).

The methods used to determine the methicillin susceptibilities of growth and peptidoglycan syntheses were essentially as described by Smith and Wilkinson (15). Experiments were carried out at 30°C to ensure a high degree of expression of methicillin resistance in strain DU4916 (15). Growth was monitored by measuring turbidity at 580 nm. Methicillin was added to early exponential-phase cultures (zero time). Peptidoglycan synthesis in actively growing cells was measured as previously described (15), except L-[4,5-H(N)]lysine (specific activity, 80.5 Ci/mmol; 1 μCi/ml) was included in the medium. To measure peptidoglycan synthesis in cell wall synthesis solution, washed exponential-phase organisms were suspended in cell wall synthesis solution containing 100 mM KH_2PO_4, 25 mM glucose, 1 mM L-alanine, 1 mM L-glutamic acid, 1 mM glycine, 0.1 mM L-lysine, 0.01 μCi of L-[4,5-H(N)]lysine per ml, 1 mM $MgCl_2$, 0.1 mM $MnCl_2$, and 20 μg of nicotinamide and 1 μg of thiamine per ml (pH 7.2). Peptidoglycan was isolated as described previously (15), and radioactivity was measured by suspension of peptidoglycan in liquid scintillation cocktail (Beckman GP) and counting in a liquid scintillation spectrometer (model LS-8100; Beckman Instruments, Inc., Palo Alto, Calif.).

Peptidoglycan synthesis in strain DU4916 was inhibited 67% by 10 μg of methicillin per ml in cell wall synthesis solution (Table 1). In contrast, peptidoglycan synthesis in actively growing cultures of strain DU4916 was inhibited by only 25% by 100 μg of methicillin per ml after 2 h. Under these conditions, peptidoglycan synthesis in strain DU4916S was inhibited by 93 and 88% with 10 μg of methicillin per ml in cell wall synthesis solution and actively growing cultures, respectively. The growth of strain DU4916 was inhibited by only 29% with 100 μg of methicillin per ml, whereas the growth of strain DU4916S was much more markedly inhibited in the presence of 10 μg of methicillin per ml (Table 1) (15).

TABLE 1. Methicillin susceptibilities of growth and peptidoglycan syntheses[a]

Strain and methicillin concn (μg/ml)	Growth (A_{580}) at:		% Inhibition	Peptidoglycan synthesis			
				Growth medium		Wall synthesis solution	
	Zero time	2 h		CPM incorporated after 2 h	% Inhibition	CPM incorporated after 2 h	% Inhibition
DU4916							
Control	0.23	0.75	0	31,399	0	37,298	0
10	NT	NT	NT	NT	NT	12,391	67
100	0.23	0.60	29	23,522	25	NT	NT
DU4916S							
Control	0.215	0.70	0	27,812	0	34,822	0
10	0.215	0.33	76	3,434	88	2,567	93

[a] A_{580}, Absorbancy at 580 nm; NT, not tested.

Electron microscopy showed that lysis of strain DU4916S had begun (see below). Although the level of peptidoglycan synthesis inhibition by methicillin in strain DU4916 in cell wall synthesis solution is somewhat less than that previously found (15), the results are consistent with the hypothesis that wall-thickening peptidoglycan synthesis is methicillin susceptible but septal peptidoglycan synthesis is methicillin resistant in the methicillin-resistant strain. Both peptidoglycan syntheses are methicillin susceptible in the methicillin-susceptible strain.

The cell populations in growth medium or cell wall synthesis solution were sampled during these experiments for examination by electron microscopy. Samples were taken at zero time just before the addition of methicillin and after 2 h of incubation in the presence or absence of methicillin.

Strain DU4916 cells at time zero in actively growing cultures (Fig. 1A) had an ultrastructure typical of *S. aureus*, with septa that were normal in appearance and a trilaminar cell wall (2). Two hours after the addition of methicillin (100 μg/ml) to an actively growing culture of strain DU4916, bizarre, grossly enlarged septa were preset in all cells in the field, and septa in different planes were seen in some of the cells (Fig. 1B and C). No cell lysis was observed. The results provide dramatic evidence that septal peptidoglycan synthesis continued in the presence of methicillin in strain DU4916. The occurrence of thickened septa and septa in more than one plane indicates that methicillin interfered with proper cell division in the methicillin-resistant strain, possibly owing to inhibition of peripheral cell wall synthesis by methicillin which thereby blocks proper cell wall growth, resulting in the diversion of excess new cell wall material to the septal region. It can be seen that 100 μg of methicillin per ml slowed the growth of the methicillin-resistant strain after 2 h as compared with the growth of control cultures (Table 1).

In the equivalent experiment in strain DU4916S, lysed, partially lysed, and swollen cells occurred in cultures treated with 10 μg of methicillin per ml (Fig. 1E) as compared with control cultures (Fig. 1D). From scanning electron micrographs, the average diameter of cells of methicillin-treated strain DU4916S was 1.39 μm, compared with a diameter of 0.87 μm in control cells. Thus, addition of methicillin to actively growing cultures of the methicillin-susceptible strain led to extensive cell lysis. This is the typical response of susceptible *S. aureus* strains to β-lactam antibiotics (12). In addition, enlarged septa could be seen in some of the cells in the preparation (Fig. 1F). These observations are similar to earlier findings in which treatment of penicillin-susceptible *S. aureus* with penicillin resulted in large cells with thinner and less regular peripheral walls and thickened, blunted, misshapen, and multiple septa (9, 10). It appears that accumulation of peptidoglycan in the septal region precedes cell lysis in the methicillin-susceptible strain.

When cells of either strain incubated in cell wall synthesis solution were examined, it was difficult to see clearly whether cell wall thickening had occurred. Cell wall synthesis was probably not of sufficient magnitude for ready detection by electron microscopy. However, in both strains, methicillin (10 μg/ml) caused the appearance of thickened septa (data not shown), perhaps by inhibiting peripheral cell wall synthesis and diverting peptidoglycan to the septal region.

From the results presented here, it appears that methicillin does not inhibit septal peptidoglycan synthesis in the methicillin-resistant *S. aureus* strain but does interfere with normal cell division, perhaps by inhibiting peripheral wall growth (3). This observation may explain why methicillin-resistant strains grow more slowly in the presence of methicillin than in its absence

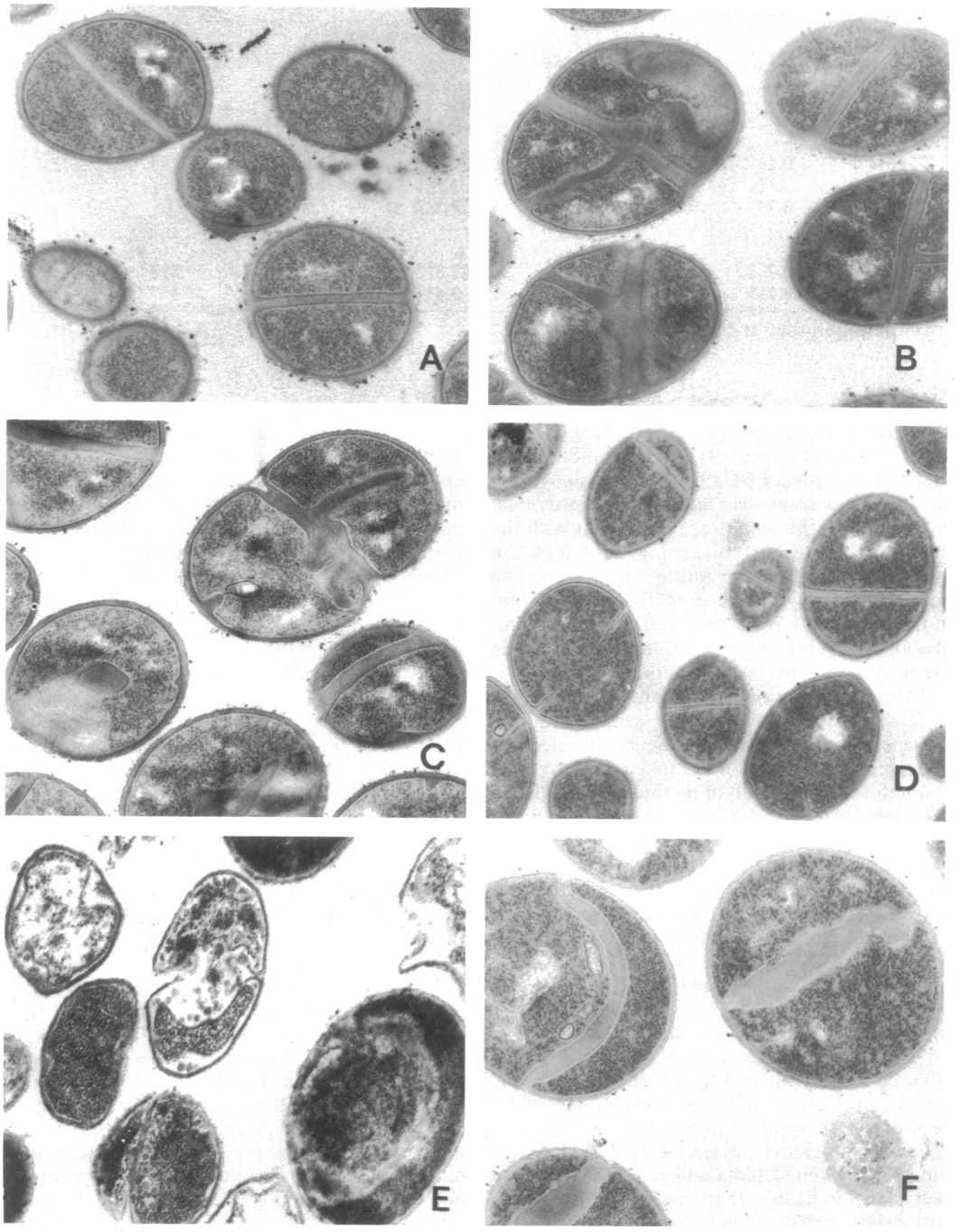

FIG. 1. Effects of methicillin on actively growing cultures of *S. aureus* strains DU4916 (A through C) and DU4916S (D through F). (A) and (D) Cells at time zero; (B) and (C) cells after 2 h of incubation in the presence of methicillin (100 μg/ml); (E) and (F) cells after 2 h of incubation in the presence of methicillin (10 μg/ml). Magnification, ×44,000. Samples for electron microscopy were fixed in phosphate-buffered 5% glutaraldehyde for 2 h, followed by buffered 1% osmium tetroxide for 2 h, and were finally fixed in aqueous 1% uranyl acetate for 1 h. The samples were dehydrated in ethyl alcohol and embedded in Epon 812.

(Table 1) (1). Recently, Rozgonyi et al. (13) have reported the occurrence of large, multiseptated cocci in cultures of a slowly growing, methicillin-resistant *S. aureus* strain in the absence of methicillin.

Three groups have reported changes in the affinities of penicillin-binding proteins (PBPs) for β-lactam antibiotics in methicillin-resistant *S. aureus* compared with methicillin-susceptible strains (1, 7, 8). PBPs are thought to correspond to various penicillin-sensitive enzymes involved in peptidoglycan metabolism (16). Although several PBPs have been identified with particular physiological functions in gram-negative, rod-shaped bacteria, such as *Escherichia coli* (16), similar assignments of functions to *S. aureus* PBPs have not been made. Two of the studies on the PBPs of methicillin-resistant *S. aureus* strains showed that, of four total PBPs, only PBP 3 had a reduced affinity for β-lactam antibiotics (1, 8). From our previous work on the differential methicillin susceptibilities of peptidoglycan syntheses, we suggested that PBP 3 may be the transpeptidase enzyme (17) involved in septum formation in *S. aureus* (15). The results presented in this paper provide further evidence in support of this idea. Recently, Wyke et al. (18) reported that growth of a methicillin-resistant strain in the presence of low concentrations of methicillin led to hypo-cross-linked peptidoglycan. They proposed that PBPs 2 and 3 can intercompensate their roles as transpeptidases and that one of these proteins acts as the primary transpeptidase for incorporation of newly synthesized peptidoglycan into the growing cell wall.

Additionally, it seems that an important component of methicillin resistance is that lysis does not occur upon treatment of methicillin-resistant strains with the drug. If the major determinant of methicillin resistance in strain DU4916 is the presence of a methicillin-resistant septal peptidoglycan synthetic system, then a further implication of this study is that interference with septal peptidoglycan synthesis in particular is important for the triggering of cell lysis in β-lactam antibiotic-susceptible strains. Similar conclusions have been made with regard to PBP 3 of *Escherichia coli* in that this PBP appears to be involved in septum formation and lysis triggering (11).

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