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Effects of Temperature, NaCl, and Methicillin on Penicillin-Binding Proteins, Growth, Peptidoglycan Synthesis, and Autolysis in Methicillin-Resistant *Staphylococcus aureus*

MURTY V. V. S. MADIRAJU,† DAVID P. BRUNNER,‡ AND BRIAN J. WILKINSON*

Microbiology Group, Department of Biological Sciences, Illinois State University, Normal, Illinois 61761

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Methicillin-resistant *Staphylococcus aureus* strains produce a fifth penicillin-binding protein (PBP), PBP 2′, with low affinity for β-lactam antibiotics that is believed to represent a β-lactam-insensitive peptidoglycan transpeptidase. In an effort to evaluate the adequacy of PBP 2′ as an explanation of methicillin resistance, PBP 2′ production and the responses of growth and peptidoglycan synthesis to methicillin under different environmental conditions have been compared. In the heterogeneous methicillin-resistant strain DU4916-K7, less PBP 2′ was produced at 40°C than at 30°C, but inclusion of 5% (wt/vol) NaCl in the medium at 40°C boosted PBP 2′ production and allowed growth of the organism in the presence of 10 μg of methicillin per ml. When exponential-phase cultures were challenged with methicillin, growth and peptidoglycan synthesis were much more resistant at 30°C than at 40°C. Inclusion of NaCl in medium rendered growth and peptidoglycan synthesis more methicillin resistant at 40°C. Hence, there was a good correlation between PBP 2′ production and methicillin-resistant peptidoglycan synthesis under these conditions. However, PBP 2′ production was increased by NaCl at 30°C without markedly affecting the susceptibilities of growth and peptidoglycan synthesis to methicillin. Pregrowth of cells with methicillin, which was expected to boost PBP 2′ production, seemed to increase the susceptibilities of growth and peptidoglycan synthesis to methicillin. Patterns of growth and peptidoglycan synthesis susceptibilities to methicillin which were similar to those described above were found in chloramphenicol-inhibited cultures, in which presumably no induction of PBP 2′ could occur during the methicillin challenge period. Complex effects were noted in the combination of subinhibitory methicillin and NaCl. Growth of cells in the presence of NaCl stimulated their autolytic activity, which was further increased by growth with subinhibitory methicillin in addition to NaCl. It appears that NaCl enhances methicillin resistance by stimulating PBP 2′ production and providing osmotic support but opposes it by stimulating autolytic activity which is exacerbated by the very low cross-linking of peptidoglycan in methicillin-resistant strains grown in the presence of methicillin.

Recent studies have shown that the production of an additional, fifth penicillin-binding protein (PBP), PBP 2′ or 2a, with low affinity for β-lactam antibiotics is intimately involved in methicillin resistance in *Staphylococcus aureus* (10, 19). Production of PBP 2′ is decreased in cells grown at pH 5.2 (10) but is increased by growth of cells at low temperatures (20, 26, 27), in the presence of NaCl (20), and in the presence of β-lactam antibiotics (5, 20, 21, 26). There is a reasonable correlation between the effects of these various environmental factors on PBP 2′ production and methicillin resistance expression (23). Recently, molecular cloning of the PBP 2′ gene in *Escherichia coli* has been reported (16).

It is generally assumed that PBP 2′ represents a penicillin-insensitive transpeptidase capable of carrying out peptidoglycan synthesis in the presence of β-lactam antibiotics (B. J. Wilkinson, M. V. V. S. Madiraju, and D. P. Brunner, *Clinical Implications of Antimicrobial Resistance*, in press), although few studies have attempted to examine this (4, 24, 31). Studies of the lytic properties (18, 29), genetics (2), and resistance expression (11) of methicillin-resistant *S. aureus* have raised the possibility that PBP 2′ production alone is not the entire explanation of methicillin resistance. Recently, Berger-Bächi et al. (3) reported that a methicillin-susceptible strain derived from a methicillin-resistant strain by insertion of transposon Tn*551* still produced PBP 2′. In this work, we have attempted to evaluate the role of PBP 2′ in methicillin resistance by comparing PBP 2′ production with the susceptibilities of growth and peptidoglycan synthesis to methicillin in cells grown under various conditions. We found a good correlation between PBP 2′ production and the methicillin resistances of growth and peptidoglycan synthesis under certain environmental conditions. The combination of NaCl and methicillin revealed unexpectedly complex effects.

MATERIALS AND METHODS

Strains and growth conditions. Methicillin-resistant, penicillinase-producing strain DU4916, the derived methicillin-resistant, penicillinase-negative strain DU4916-K7, and the methicillin-susceptible, penicillinase-negative strain DU49 16S were used (8, 18). For study of PBPs, the strains were grown with shaking in PYK medium as previously described (18) with and without NaCl and methicillin supplementation at 30 and 40°C from a 1% (vol/vol) inoculum from an overnight culture in PYK medium at 30°C to mid-exponential phase, i.e., an A_{580} of about 0.7.

Methicillin MICs and population analyses. MICs were determined by tube dilution as described previously (24). Population analyses were done by determining the efficiency

* Corresponding author.
† Present address: Department of Molecular Biology, University of California-Berkeley, Berkeley, CA 94720.
‡ Present address: The Upjohn Company, Kalamazoo, MI 49001.

TABLE 1. Methicillin MICs and population analyses (EOP) under different conditions

Strain	Determinations at:											
	30°C			30°C with NaCl			40°C			40°C with NaCl		
	MIC[a]	EOP[b] at μg/ml		MIC	EOP at μg/ml		MIC	EOP at μg/ml		MIC	EOP at μg/ml	
		10	25		10	25		10	25		10	25
DU4916	>1,600	170	103	12.5	104	75	50	103	79	50	86	70
DU4916-K7	1,600	0.65	<0.12	1.56	87	11.6	3.12	<0.05	<0.05	12.5	<0.11	<0.11

[a] MIC (μg/ml) after 48 h. The values are the averages of three separate determinations.
[b] Percent of population forming colonies on methicillin-containing agar (10 or 25 μg/ml) compared with drug-free agar after 48 h. The values are the averages of two separate determinations.

of plating (EOP) by comparing colony counts on methicillin-containing agar to those on drug-free agar (24).

Studies of PBPs. PBPs were studied in membranes and whole cells by using phenyl-4(*n*)-[^{3}H]benzylpenicillin, ammonium salt (15 to 30 Ci/mmol; Amersham Corporation, Arlington Heights, Ill.) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography as previously described (18, 20). Equal amounts of protein from samples under the different growth conditions were loaded on the gels. A 10% acrylamide–0.068% bisacrylamide gel was used, and electrophoresis was continued for an additional 200 V·h after the dye front reached the bottom of the gel.

Susceptibilities of growth and peptidoglycan synthesis to methicillin in exponential phase and chloramphenicol-inhibited cultures. To radiolabel peptidoglycan, D-[1,6-^{3}H(*N*)] glucosamine (32.5 Ci/mmol; 0.5 μCi/ml; New England Nuclear Corp., Boston, Mass.) and 100 μM unlabeled D-glucosamine were included in the medium. Growth was initiated with a 1% (vol/vol) inoculum from a culture grown overnight in PYK medium at 30°C. Growth was monitored at A_{580}, and when the culture reached an A_{580} of 0.2 to 0.3, methicillin was added. Samples (1 ml) were removed at intervals and mixed with 3 ml 10% (wt/vol) trichloroacetic acid at 4°C for isolation of peptidoglycan by a procedure based upon that of Mychaljonka et al. (17) (V. L. Hicks and B. J. Wilkinson, manuscript in preparation). Briefly, samples were heated for 20 min in a boiling water bath and were filtered onto 0.45-μm Whatman 934 AH glass fiber filter disks (Reeve Angel, Whatman, Inc., Clifton, N.J.). The disks were washed with cold 10% (wt/vol) trichloroacetic acid, 75% (vol/vol) ethanol, and 0.01 M $NaPO_4$ buffer (pH 8.0), followed by trypsin digestion and further washing. Radioactivity was determined by scintillation counting.

Peptidoglycan synthesis and its response to methicillin was also measured in chloramphenicol-inhibited cultures. Chloramphenicol (100 μg/ml) was added to cultures when they reached an A_{580} of 0.2 to 0.3, and 1 h later, methicillin was added.

Measurement of autolysis. The strains were grown to mid-exponential phase in media with various experimental additions at 30°C, and autolysis of washed cells was measured at 30°C in 0.05 M KPO_4 buffer (pH 7.2) as described by Qoronfleh and Wilkinson (18).

RESULTS

Characteristics of strain DU4916-K7: MICs and population analyses. Strain DU4916 is a relatively extensively studied, homogeneous, methicillin-resistant strain (8, 15, 18, 24, 29), and strain DU4916-K7 is a less-well-studied derivative produced by acriflavine treatment of strain DU4916 (8). In Table 1, MICs and population analyses of strains DU4916 and DU4916-K7 under different growth conditions are shown. Both strains were highly resistant to methicillin at 30°C but their MICs were much lower at 40°C. For both strains, the MICs at 30°C with NaCl were much lower than in the absence of NaCl. This large disparity between MICs with and without NaCl was not seen at 40°C. Strain DU4916-K7 was somewhat less resistant than strain DU4916, as revealed by the data in Table 1 and by the observation that its 24-h MICs were lower at 30°C (data not shown).

Strain DU4916 showed more homogeneous methicillin resistance than strain DU4916-K7, in that the EOP of strain DU4916 was high under all conditions examined. Strain DU4916-K7 behaved as a heterogeneous resistant strain in that its EOP was markedly influenced by temperature and NaCl. Evidently, strain DU4916-K7 has lost some homogeneous resistance trait as well as penicillinase production compared with strain DU4916. It is necessary that a strain be a non-penicillinase producer for PBP assays.

Strain DU4916S was highly methicillin susceptible (MICs of 1.56 or 3.12 μg/ml) under all conditions.

Influence of growth conditions on PBP profiles. The PBP profiles of strain DU4916-K7 under different growth conditions are shown in Fig. 1a. Large amounts of PBP 2′ were produced in strain DU4916-K7 cultures grown at 30°C (lane 2), 30°C with 5% (wt/vol) NaCl (lane 3), 30°C with methicillin (10 μg/ml) (lane 4), and 30°C with NaCl plus methicillin (lane 5). This protein is not produced by the methicillin-susceptible strain DU4916S (lane 1). PBP 3 was not detected in cells grown at 30°C with methicillin either with or without NaCl (lanes 4 and 5). This absence of PBP 3 may be due to saturation of PBP 3 with methicillin during growth, thus rendering it undetectable. Reynolds and Brown (20) noted an apparent severe depression in the level of PBP 3 in strain DU4916-K7 grown in the presence of methicillin and suggested that methicillin may have inhibited the synthesis of this PBP. The level of PBP 2′ was dramatically reduced in strain DU4916-K7 grown at 40°C (lane 7), and PBP 2 was not detected. Inclusion of NaCl in the medium boosted production of PBP 2′ at 40°C (lane 8), and NaCl presence in the medium was necessary to allow growth at 40°C with methicillin (10 μg/ml). PBP 2 was not detected in strain DU4916S grown at 40°C (lane 6). Decreased detection of PBP 2 at 43°C has been noted by Utsui and Yokota (27). Thus, production of PBP 2′ was decreased by elevated temperature and increased by NaCl, in accord with the findings of Reynolds and Brown (20). Inclusion of methicillin in the medium did not appear to have much influence on PBP 2′ production. However, our experimental conditions might not have been ideal for observing this production. For example, PBP 2′ may be partially saturated by inclusion of methicillin in the growth medium, thus reducing the amount of [^{3}H]benzylpenicillin bound in the PBP assay. Reynolds

and Brown (20) exposed strain DU4916-K7 to methicillin and then cultivated it in the absence of methicillin for 5 generations before assaying PBPs, presumably to increase detection of PBP 2′. They reported that PBP 2′ was increased by growth in the presence of methicillin.

In Fig. 1b, the Coomassie blue-stained gel of the membrane preparations from which the fluorograph (Fig. 1a) was made is shown. A protein of 78,000 M_r, believed to represent PBP 2′, is seen in the same lanes (lanes 3 to 6 and 9) as in Fig. 1a. There is more of the protein in cultures grown at 30°C than in those grown at 40°C, and NaCl stimulates its production. These data tend to confirm the conclusions made about PBP 2′ levels on the basis of fluorography.

The fact that PBP 2′ has a low affinity for methicillin was demonstrated by incubating isolated membranes with methicillin before labeling with [^{3}H]benzylpenicillin (Fig. 2). PBP 2′ was not fully saturated by 5 mg of methicillin per ml, whereas PBP 3 and PBP 2 were saturated by 10 and 50 μg of methicillin per ml, respectively. The concentrations of methicillin chosen as challenge doses (100 and 1,000 μg/ml)

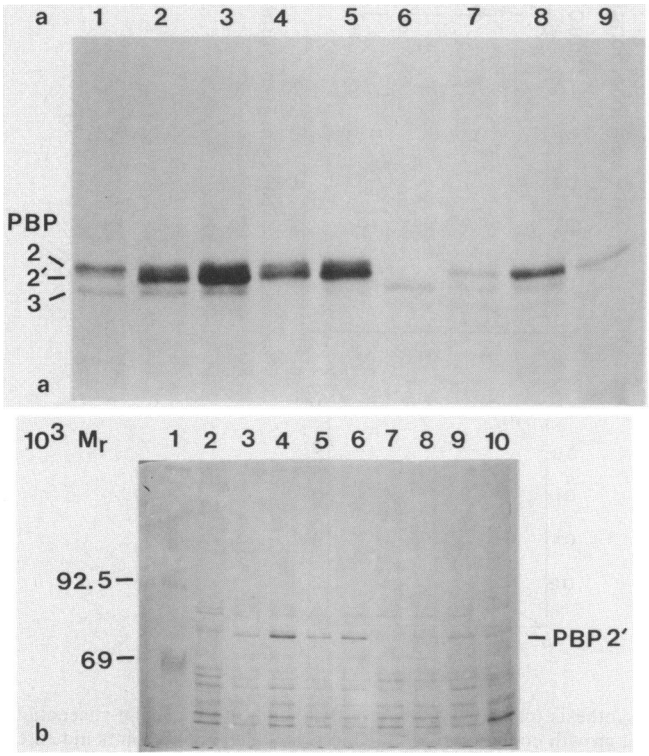

FIG. 1. (a) Penicillin-binding protein profiles of methicillin-susceptible strain DU4916S and methicillin-resistant strain DU49 16-K7 grown under different conditions. Membranes were isolated from the organisms grown under the different conditions and were incubated with [^{3}H]benzylpenicillin, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography to reveal PBPs. Lanes: 1, DU4916S at 30°C; 2, DU4916-K7 at 30°C; 3, DU4916-K7 at 30°C with NaCl (5% wt/vol); 4, DU4916-K7 at 30°C with methicillin (10 μg/ml); 5, DU4916-K7 at 30°C with NaCl (5% wt/vol) and methicillin (10 μg/ml); 6, DU4916S at 40°C; 7, DU4916-K7 at 40°C; 8, DU4916-K7 at 40°C with NaCl (5% wt/vol); 9, DU4916-K7 at 40°C with NaCl (5% wt/vol) and methicillin (10 μg/ml). (b) Membrane protein profiles of methicillin-susceptible strain DU4916S and methicillin-resistant strain DU4916-K7 grown under different conditions. The Coomassie-blue stained gel from which the fluorogram shown in Fig. 1a was prepared is shown. Lane 1, M_r markers; for lanes 2 through 10, see legend for lanes 1 through 9 in Fig. 1a.

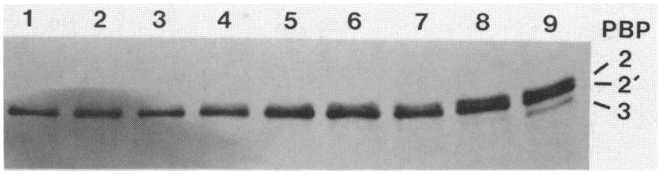

FIG. 2. Competition of methicillin for PBPs of strain DU49 16-K7. The organism was grown at 30°C for preparation of membranes. The membranes were preincubated with various concentrations of methicillin for 10 min at 30°C before incubation with [^{3}H]benzylpenicillin. Lanes show methicillin at 5,000 (lane 1), 1,000 (lane 2), 500 (lane 3), 100 (lane 4), 50 (lane 5), 10 (lane 6), 5 (lane 7), 1 (lane 8), and 0 μg/ml (lane 9).

in studies of growth and peptidoglycan synthesis were expected to saturate all PBPs except PBP 2′ in strain DU49 16-K7.

Responses of growth and peptidoglycan synthesis to methicillin under different environmental conditions. The results of experiments to measure growth and peptidoglycan synthesis are shown in Fig. 3 and are summarized in Table 2. Growth and peptidoglycan synthesis are plotted arithmetically rather than semilogarithmically, because the data are more clearly presented in this way for the portions of the growth curve shown. As expected, growth and peptidoglycan synthesis were substantially inhibited by methicillin in methicillin-susceptible strain DU4916S at either temperature. Growth and peptidoglycan synthesis by strain DU49 16-K7 were markedly more susceptible to methicillin at 40°C than at 30°C. More PBP 2′ was produced at 30°C than at 40°C (Fig. 1a and b). Growth appeared to be somewhat more resistant and peptidoglycan synthesis was somewhat more susceptible to methicillin in strain DU4916-K7 grown with NaCl at 30°C than in cells grown at 30°C. NaCl slowed the growth of the cells to some extent (compare Fig. 3c and e and d and f). More PBP 2′ appeared to be produced at 30°C with NaCl than without NaCl. At 40°C, NaCl increased PBP 2′ production, and growth and peptidoglycan synthesis were more resistant to methicillin than were those of cells grown at 40°C without methicillin. Prior growth of cells with methicillin (10 μg/ml) at 30°C before challenge with methicillin had little effect on the growth rate of the culture compared with that of cells grown without the antibiotic. However, both growth and peptidoglycan synthesis appeared to be somewhat more methicillin susceptible in these cultures than in cultures grown without methicillin at 30°C with or without NaCl (Table 2). Thus, conditions that might have been expected to dramatically increase PBP 2′ production did not give highly resistant cells. The cells were unable to grow at 40°C with methicillin (10 μg/ml). Cells growing at 30°C with NaCl and methicillin were expected to have maximal induction of PBP 2′, and growth and peptidoglycan synthesis appeared to be somewhat more methicillin resistant than under the other cultural conditions. However, even in the control culture (i.e., the culture not challenged with additional methicillin), the cells were growing very slowly, and later in the growth cycle, lysis of the cultures occurred (data not shown). This finding suggested that the interplay of NaCl and methicillin on growth was complex, and we decided to study this further (see below).

Susceptibility of peptidoglycan synthesis to methicillin under different environmental conditions in chloramphenicol-inhibited cultures. The data of Rossi et al. (21) showed that PBP 2′ could be induced rapidly upon exposure of a culture to methicillin. It is possible that the susceptibilities of growth

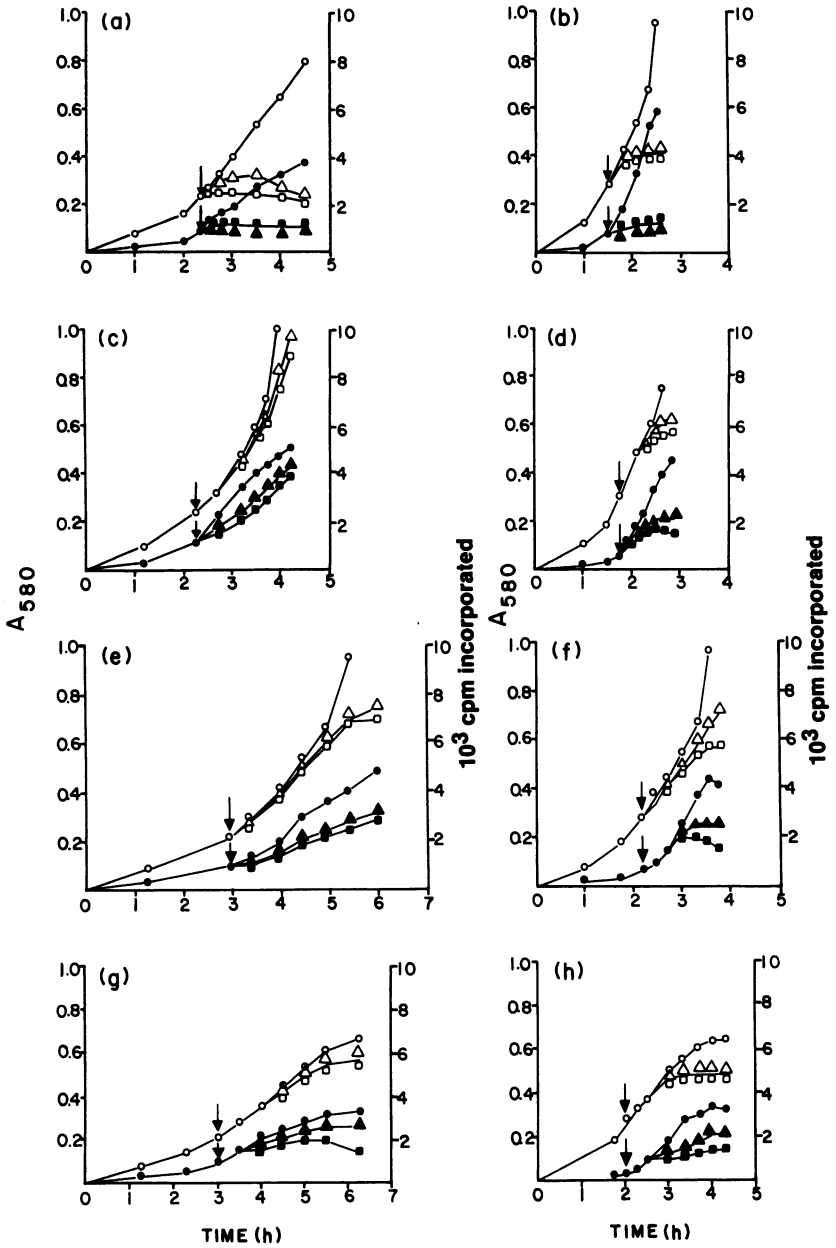

FIG. 3. Susceptibilities of growth (open symbols) and peptidoglycan synthesis (closed symbols) to methicillin in methicillin-susceptible strain DU4916S and methicillin-resistant strain DU4916-K7 under different growth conditions. Methicillin was added at the points indicated by the arrows to the cultures growing under the various conditions. ○ ●, Control; △ ▲, methicillin (10 μg/ml with methicillin-susceptible strain DU4916S, 100 μg/ml with strain DU4916-K7); □ ■, methicillin (100 μg/ml with strain DU4916S, 1,000 μg/ml with strain DU4916-K7). (a) DU4916S at 30°C; (b) DU4916S at 40°C; (c) DU4916-K7 at 30°C; (d) DU4916-K7 at 40°C; (e) DU4916-K7 at 30°C with NaCl (5% wt/vol); (f) DU4916-K7 at 40°C with NaCl (5% wt/vol); (g) DU4916-K7 at 30°C with NaCl (5% wt/vol) and methicillin (10 μg/ml); (h) DU4916-K7 at 40°C with NaCl (5% wt/vol) and methicillin (10 μg/ml).

and peptidoglycan synthesis were reflecting induction of PBP 2′ during the methicillin challenge in actively growing cultures. Inhibition of protein synthesis by chloramphenicol should "freeze" the PBP complement of the cell, preventing induction of PBP 2′ production by methicillin (5). The results of these experiments are shown in Table 3. The results show a similar pattern to those in actively growing cultures. Peptidoglycan synthesis was markedly more methicillin susceptible at 40°C than at 30°C, and inclusion of NaCl in the medium at 40°C rendered peptidoglycan synthesis more methicillin resistant. Peptidoglycan synthesis in cultures grown at 30°C with methicillin was more methicillin susceptible than in cultures grown without methicillin and with or without NaCl at 30°C. Cultures grown with NaCl and methicillin showed low incorporation of radioactivity (peptidoglycan synthesis), making the methicillin susceptibility results difficult to interpret.

Influence of NaCl and methicillin on autolysis. The apparently deleterious effect of the combination of NaCl and methicillin on growth of the methicillin-resistant strain prompted us to study this phenomenon further. Subinhibitory concentrations of methicillin decrease peptidoglycan

cross-linking through inhibition of PBP 4 (18, 31), which leads to cell walls with an increased potential for lysis (18). When either strain DU4916S or DU4916-K7 was grown in PYK medium plus NaCl, the cells autolysed more rapidly than cells grown without NaCl (Fig. 4). When strain DU4916-K7 was grown with NaCl and methicillin, the cells autolysed most rapidly (Fig. 4). This result would appear to be due to the combination of the stimulatory effect of NaCl on autolysis and the hypo-cross-linking of peptidoglycan resulting from methicillin presence during growth.

DISCUSSION

Methicillin-resistant strain DU4916-K7 cultures were more susceptible to the inhibitory effects of methicillin on growth and peptidoglycan synthesis at 40°C than at 30°C. More PBP 2′ was produced at 30°C than at 40°C. Cultivation of the cells at 40°C with NaCl increased the resistances of growth and peptidoglycan synthesis to methicillin and increased the production of PBP 2′. The cells would not grow at 40°C with 10 μg of methicillin per ml unless NaCl was present in the medium. The response of peptidoglycan synthesis to methicillin in chloramphenicol-inhibited cultures, in which no induction of PBP 2′ production by the challenging methicillin can occur (5), showed the same pattern. However, the peptidoglycan synthesis occurring in chloramphenicol-inhibited cultures is believed to represent wall thickening rather than septal peptidoglycan synthesis (24, 29). Nevertheless, these results provide supporting evidence for the supposition that PBP 2′ represents a β-lactam-insensitive peptidoglycan transpeptidase which allows cells to grow in the presence of β-lactams. Peptidoglycan synthesis was more resistant to β-lactams in a methicillin-resistant strain grown under conditions favorable for resistance expression (30°C with NaCl) than under unfavorable conditions (40°C) (4). Hartman and Tomasz (10) reported that growth and peptidoglycan synthesis rapidly became methicillin susceptible when a heterogeneously resistant strain was switched from 30 to 37°C, whereas this did not happen in a homogeneously resistant derivative.

TABLE 2. Susceptibilities of growth and peptidoglycan synthesis to methicillin in methicillin-resistant strain DU4916-K7 under different conditions

Growth conditions	Methicillin (μg/ml)	Growth (A_{580})			% Inhibition of peptidoglycan synthesis[a]	
		0 h	1 h	2 h	1 h	2 h
30°C	0	0.24		1.2		0
	100	0.24		1.0		18
	1,000	0.24		0.9		24
30°C with NaCl	0	0.22		0.67		0
	100	0.22		0.60		36
	1,000	0.22		0.59		46
30°C with methicillin (10 μg/ml)	0	0.21		1.50		0
	100	0.21		0.64		50
	1,000	0.21		0.56		50
30°C with NaCl and methicillin (10 μg/ml)	0	0.20		0.56		0
	100	0.20		0.55		8
	1,000	0.20		0.54		23
40°C	0	0.31	1.15		0	
	100	0.31	0.61		60	
	1,000	0.31	0.58		75	
40°C with NaCl	0	0.29	0.61		0	
	100	0.29	0.54		37	
	1,000	0.29	0.52		63	
40°C with NaCl and methicillin (10 μg/ml)	0	0.29	0.64		0	
	100	0.29	0.49		42	
	1,000	0.29	0.49		64	

[a] % Inhibition of peptidoglycan synthesis = 100 − [(cpm + methicillin 1 or 2 h − cpm 0 h/cpm − methicillin 1 or 2 h − cpm 0 h) × 100].

TABLE 3. Susceptibility of peptidoglycan synthesis to methicillin in chloramphenicol-treated cultures of methicillin-resistant strain DU4916-K7 grown under different conditions

Growth conditions	Methicillin (μg/ml)	% Inhibition of peptidoglycan synthesis	
		2 h	4 h
30°C	100	30	30
	1,000	53	54
30°C with NaCl	100	38	38
	1,000	52	63
30°C with methicillin (10 μg/ml)	100	69	72
	1,000	70	69
30°C with NaCl and methicillin (10 μg/ml)	100	38	42
	1,000	38	42
40°C	100	76	74
	1,000	97	97
40°C with NaCl	100	28	38
	1,000	65	79
40°C with NaCl and methicillin (10 μg/ml)	100	38	42
	1,000	38	42

At 30°C, growth with NaCl clearly increased PBP 2′ production but peptidoglycan synthesis was no more methicillin resistant. Pregrowth with subinhibitory methicillin at 30°C, which has been reported to increase PBP 2′ production in this strain (20), did not increase the resistance of peptidoglycan synthesis to methicillin. This finding is perhaps particularly puzzling, because presumably only the more resistant members of the population would be able to grow in the presence of methicillin. The explanation of this lack of correlation between PBP 2′ production and the responses of growth and peptidoglycan synthesis to methicillin is currently unclear. Hartman and Tomasz (10) have suggested that PBP 2′ may be detectable as a PBP but may be enzymatically nonfunctional in the case of heterogeneous methicillin-resistant strains producing PBP 2′ but not expressing methicillin resistance.

PBP 2′ is not saturated by 5 mg of methicillin per ml. Reynolds and Brown (20) reported that 60 μg of benzylpenicillin does not saturate the protein. Clearly, the protein is not saturated at the MIC, yet growth is inhibited. However, perhaps a sufficient fraction of the presumed transpeptidase activity of PBP 2′ is inhibited at the MIC to inhibit growth. Far fewer organisms are used in an MIC determination than in a PBP affinity determination. In the growth experiments described in this paper (Fig. 3), high cell densities were used and the strain seemed able to tolerate high methicillin concentrations as growth was markedly slowed but did not stop. Above about 10 μg of methicillin per ml, only PBP 2′ would appear to be functioning. However, such cells were not growing normally. Their peptidoglycan was poorly cross-linked (18, 31), their septa were enlarged, and their division was aberrant (29). Perhaps growth eventually stops because the normal synthesis and maturation of peptidoglycan is interfered with, even though PBP 2′ may be capable of synthesizing more peptidoglycan. Alternatively, other cellu-

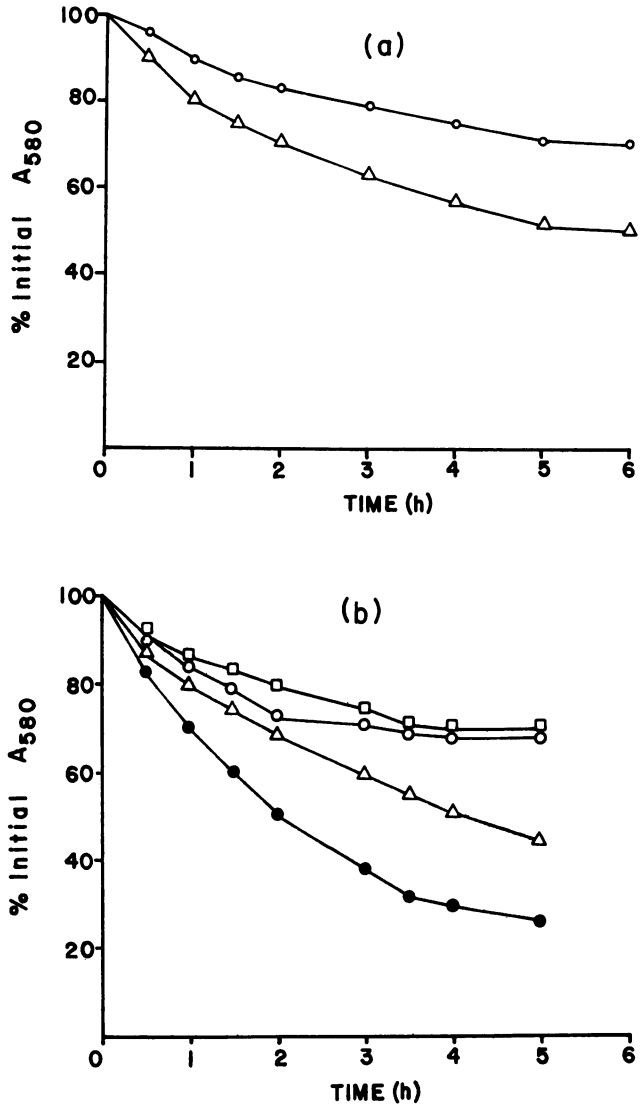

FIG. 4. Effect of growth in the presence of NaCl and methicillin on autolysis. (a) Strain DU4916S. ○, Grown in medium with no additions; △, grown with 5% (wt/vol) NaCl. (b) Strain DU4916-K7. ○, Grown in medium with no additions; △, grown with 5% (wt/vol) NaCl; □, grown with methicillin (10 μg/ml); ●, grown with NaCl and methicillin. Growth and measurement of autolysis were carried out at 30°C.

lar targets may be inhibited at high β-lactam concentrations. Rozgonyi et al. (22) have suggested that phospholipid synthesis may be a β-lactam target in *S. aureus*.

The influence of NaCl on methicillin resistance expression is complex, and NaCl has a variety of physiological effects on *S. aureus*. We have found that NaCl often decreased the MICs of methicillin for a variety of *S. aureus* and *Staphylococcus epidermidis* strains but increased their EOP on methicillin-containing agar (Table 1; 30; V. L. Hicks, M. H. Hoe, M. S. Tuscan, D. P. Brunner, and B. J. Wilkinson, unpublished observations). We acknowledge that our finding of lower methicillin MICs in NaCl-containing medium is in contrast to the currently accepted opinion of the influence of NaCl on methicillin resistance. Addition of NaCl to Mueller-Hinton broth (1) or cation-supplemented Mueller-Hinton broth (25) is recommended for detection of methicillin-resistant *S. aureus* in microdilution susceptibility tests. Nevertheless, Wilkinson et al. (28) found that NaCl sensitized some strains of methicillin-resistant *S. epidermidis* to methicillin, and Hewitt et al. (12) commented that some batches of methicillin-salt agar were relatively inhibitory for methicillin-resistant *S. aureus*. NaCl effects may be heavily medium influenced. All of our experiments were done in PYK medium. However, inclusion of NaCl in the medium allowed strain DU4916-K7 to grow in shaken cultures at 40°C in the presence of methicillin whereas no growth was obtained in the absence of NaCl. NaCl also increased the production of PBP 2′. It is not clear why PBP 2′ production responds to increased NaCl (perhaps because of osmolarity) or at what level (e.g., transcription, translation, integration into the membrane) production is increased. Perhaps PBP 2′ production increases to compensate for environmental stresses which are potentially damaging to peptidoglycan such as subinhibitory methicillin or autolysis stimulated by NaCl.

Both methicillin-susceptible and methicillin-resistant strains grown in the presence of 5% (wt/vol) NaCl autolyzed more rapidly than when grown in the absence of NaCl. Cripps and Work (7) reported that cultures grown in the presence of 4% (wt/vol) NaCl showed increased lysis. NaCl activated the autolytic activity of a teichoic acid-deficient *S. aureus* mutant (9). In electron microscope studies, it was shown that NaCl induced cell wall thinning in *S. aureus* (6). NaCl at 5% (wt/vol) (0.86 M) concentration obviously provides osmotic support for the cells and influences lipid composition (13, 14; M. S. Tuscan, D. P. Brunner, and B. J. Wilkinson, unpublished observations). We can propose that the following opposing effects are at work in methicillin-resistant *S. aureus*. Methicillin resistance is favored by the apparent stimulation in production of PBP 2′ by NaCl and by the osmotic support it provides. This effect is opposed by the activation of autolytic enzymes, especially in the presence of methicillin, in which peptidoglycan becomes severely hypocross-linked.

In summary, we have shown that there is a reasonable correlation between PBP 2′ production and the susceptibilities of growth and peptidoglycan synthesis to methicillin under certain growth conditions. Under other growth conditions, this correlation does not hold as well, which provides further evidence that PBP 2′ production is not the entire explanation of methicillin resistance. The effects of NaCl were found to be unexpectedly complex.

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