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Staphylococcal Methicillin Resistance Expression Under Various Growth Conditions

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To improve the detection of methicillin resistant staphylococci, lowered incubation temperature (30°C) and inclusion of sodium chloride in media have been empirically recommended. However, in this study, we found that sodium chloride in Peptone-Yeast Extract-K_2HPO_4 (PYK) medium decreased methicillin minimum inhibitory concentrations. Divalent cations were shown to restore the expression of staphylococcal methicillin resistance. However, when it was determined by efficiency of plating, sodium chloride increased methicillin resistance expression on agar medium in which higher divalent cations were contained in the agar medium. The decrease of minimum inhibitory concentrations at 30°C by sodium chloride occurred in Brain Heart Infusion but did not occur in other media investigated. Interestingly, both PYK and Brain Heart Infusion media had peptone, which contain cholic acids having detergent activities. Inclusion of sodium chloride in PYK caused a higher rate of autolysis. Penicillin binding protein 2a that has a low affinity to beta-lactam antibiotics, was highly inducible in methicillin resistant *Staphylococcus epidermidis* strains. In this study, we found that autolysins that are activated by the sodium chloride decreased the minimum inhibitory concentration at 30°C, and peptidoglycan is weakened due to the presence of methicillin. Peptone in the media may aggravate the fragile cells. However, stabilization due to the presence of divalent cations and production of penicillin binding protein 2a increase the survival of staphylococci.

Key words: Methicillin, Staphylococcus, penicillin binding proteins, divalent cations, peptidoglycan.

Methicillin is a semi-synthetic penicillin that was designed to withstand the action of staphylococcal penicillinase. Methicillin inhibits bacterial growth by inhibiting a set of penicillin-binding proteins (PBPs) that catalyse the final stages of peptidoglycan synthesis. In some staphylococcal strains methicillin resistance was shown to develop. In order to improve the detection of methicillin resistance, altered cultural conditions such as increased medium NaCl concentrations, lowered temperatures, and increased incubation times have been empirically derived (2, 7, 12). Methicillin resistance is not due to drug inactivation by beta-lactamases (penicill- inase) but by reduction in the affinity of PBPs for beta-lactam antibiotics. Resistant strains produce an additional 78-kilodalton PBP, termed PBP2a, that has low binding affinity for beta-lactam antibiotics (1, 8, 9, 17). Presumably, in the presence of penicillin, PBP 2a can substitute for essential PBPs and can perform the functions necessary for cell wall assembly (19). However, the mechanism of methicillin resistance is not fully understood. Resistance expression is a complex, multifactorial interaction involving the organism, antibiotic, and the physical and chemical growth environment (15). For a proposed mechanism to be satisfactory, the variable expression of resistance depending upon various growth conditions must be accounted for. In this paper some of these complex interactions are studied; and some insights into the expression of methicillin resistance at the cellular level are reported.

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Materials and Methods

Bacterial strains and growth conditions

Staphylococcus epidermidis methicillin-resistant strains II-5, II-17 and II-47, methicillin-susceptible strains ATCC 14990, 3a, and *Staphylococcus aureus* homogeneously resistant strain DU4916 and heterogeneously resistant DU4916-K7 were studied. Organisms were stored at 4°C following growth at 37°C for 24 h on Tryptic Soy Agar (Difco) slants. The various kinds of media used are described below, and Bacto Agar (15 g/l) was added when solid

medium was required. The pH of the media, adjusted by the addition of H_2SO_4, was 7.2 except where otherwise noted.

Growth media

PYK medium was composed of 5.0 g of Bacto Peptone (Difco), 5.0 g of yeast extract (Difco) and 3.0 g of K_2HPO_4 per liter of distilled water. Five percent (w/v) NaCl and divalent cations (Mg^{++}, Ca^{++}) were added when needed. Stock solutions of $MgCl_2$ and $CaCl_2$ were filter sterilized using a 0.45 υm Millipore filter and stored at 4°C. Media without divalent cations were autoclaved and cooled in an ice bath. And $CaCl_2$ and $MgCl_2$ from the stock solutions were added to medium to yield final concentrations of 50 and 25 μg/ml, respectively. The medium was stored at 4°C until use. Tryptic Soy Broth (TSB), Brain Heart Infusion (BHI), and Mueller-Hinton (M.-H.) were used according to the manufacturer's recommendations.

Determination of methicillin minimum inhibitory concentrations (MICs)

The MIC is the lowest concentration of methicillin that inhibits growth of the organisms. The dilution method was used to determined the MICS. Methicillin was dissolved in the respective broth at a concentration of 3200 μg/ml with corrections for the potency and was sterilized using a 0.45 μm Milipore filter. A series of two fold dilutions ranging from 1600 μg/ml to 1.56 μg/ml of methicillin were made, and inoculated with 0.5 ml of a 1:2000 dilution of an overnight culture. The methicillin MICs for the strains were determined under various conditions after 48 h.

Population analyses (efficiency of plating, EOP)

Population analyses were performed by comparing colony counts in the presence and absence of methicillin (50 μg/ml) using solid medium. An overnight culture without methicillin was serially diluted. And 0.1 ml aliquotes of the 10^{-5}, 10^{-6}, and 10^{-7} dilutions were plated in triplicate and incubated at 30°C for 48 h. The percent methicillin resistance expression was calculated using the following equation: colony forming units (CFU) on PYK with methicillin × 100/CFU on PYK without methicillin.

Autolysis

A start culture was inoculated with bacteria and grown overnight at 30°C. Various media were inoculated and grown to an A_{580} nm of 0.7 at 30°C and 42°C. Fifty milliliters of culture were harvested and washed in 0.05 M KH_2PO_4 buffer (pH 7.2). The washed cells were resuspended in 50 ml of 0.05 M KH_2PO_4 buffer and incubated with shaking(200 rpm). And the A_{580} nm was measured at intervals.

Penicillin-binding proteins (PBPs)

Membranes used in PBPs assays were isolated as described by Madiraju *et al.* (14) and Singh *et al.* (16). Protein concentration was estimated as described by Lowry *et al.* (1951). Beta-lactamase-producing strains were determined with CEFINASE Tm discs (BBL). And membranes were preincubated with the beta-lactamase inhibitor clavulanic acid (50 μg/ml) for 3 min at 30°C prior to addition of radiolabeled phenyl-4(n)-^{3}H-benzyl penicillin (Amersham International Plc Amersham, U. K.) (5, 19). The preservative of an appropriate amount of ^{3}H-benzylpenicillin was removed with a gentle stream of N_2 gas and redissoved in 100 μl of benzylpenicillin (1 μg/μl) to give 5 μCi/ug. Membranes (50 μg) were placed in a tube and 47.5 μl of 0.05 M Tris-HCl-$MgCl_2$ buffer (pH 7.2) was the tube followed by 2.5 μl of ^{3}H-penicillin. The mixture was incubated at 30°C for 15 min in a shaking water bath. The reaction was stopped by adding 5 μl of benzylpenicillin (120 mg/ml) and boiled for 3 min. SDS-PAGE was carried out using 20~50 μg protein by the method of Laemmli and Favre (11).

Fluorography

After staining and destaining, the gel was soaked for 20 min in dimethyl sulfoxide, followed by a second 20 min immersion in fresh dimethyl sulfoxide. And then soaked in 22.2%(wt/vol) 2,5-diphenyloxazole in dimethyl sulfoxide for at least 2 h. The gel was rinsed with water and was soaked in 20 volumes of water for at least 1 h to remove the dimethyl sulfoxide before drying under a vacuum. Kodak X-ray film was placed with the dried gel and exposed at -70°C for 3 weeks.

Results and Discussion

Minimum inhibitory concentrations (MICs)

It is believed that NaCl in medium enhances the expression of methicillin resistance and thereby increases the frequency of detection of methicillin resistant staphylococci. However, in this study, we did not expect to find that MICs were lower in PYK+NaCl medium than in PYK medium (Fig. 1A). In strain DU4916-K7, this decrease in MIC was particularly striking. Laboratory media are often deficient in divalent cations (10), and cation supplementation is recommended for antimicrobial sus-

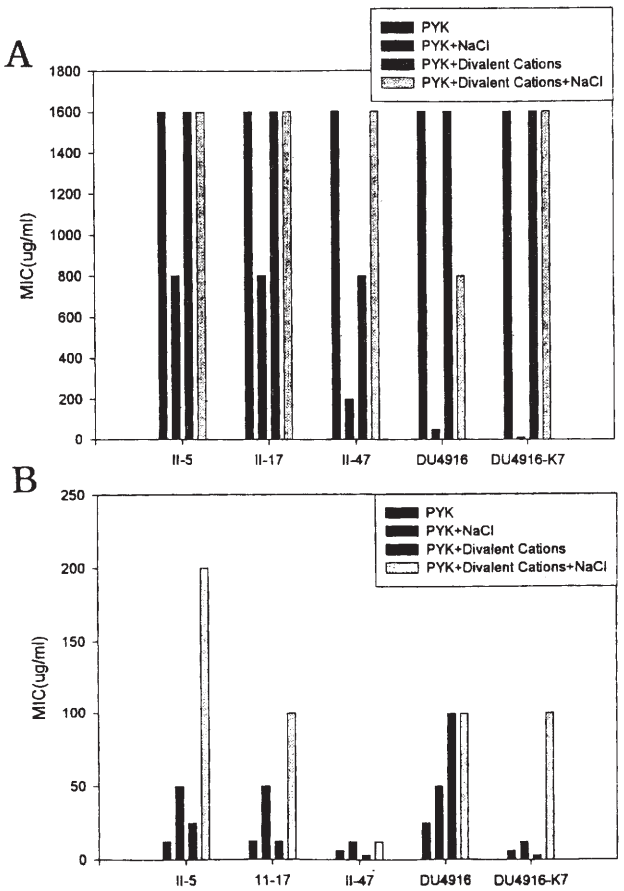

Fig. 1. Influence of NaCl and divlalent cations on methicillin MICs in PYK media at 30°C (A) and at 40°C (B). Addition of NaCl lowered the MICs at 30°C. Supplementation of divalent cations restored the MICs. Methicillin MICs at 40°C were much lower than at 30°C. The decrease of MICs by NaCl did not occur at 40°C.

ceptibility testing (18). Addition of divalent cations had small effects on MIC values of PYK medium; however, divalent cations restored MIC values in PYK+NaCl medium. Methicillin MICs in PYK medium were much lower at 40°C than at 30°C (Fig. 1B). The phenomenon of MIC decrease by NaCl was not seen at 40°C. The decrease of MICs by NaCl at 30°C did not occur in Mueller-Hinton (H.-H.) or Tryptic Soy broth (TSB) media, but occurred in Brain Heart Infusion (BHI) medium (Fig. 2). Lowering of MIC by NaCl is a medium-dependent phenomenon. TSB had markedly higher divalent cation contents than PYK medium, but M.-H. and BHI had similar divalent cation levels to those of PYK medium (Table 1). Interestingly, both media (PYK and BHI) in which MICs were decreased by NaCl contain peptone, which has cholic acids having detergent activities. The peptone may contribute to the decrease of MICs in staphylococci.

Table 1. Content of divalent cations of various media when constituted

Medium	Cation concentration (mM)	
	Ca^{++}	Mg^{++}
PYK	0.09	0.20
PYK agar	1.85	0.94
M.-H.	0.24	0.39
TSB	0.55	0.69
BHI	0.07	0.34

Efficiency of platings (EOPs)

Methicillin resistance was determined with population analyses (efficiency of plating, EOP). EOP values were higher on PYK+NaCl agar medium than PYK agar medium at 30°C (Fig. 3). Inclusion of NaCl increased the EOP of strains II-17, II-47 and DU4916-K7, with the exception of II-5 where the EOP was similar in both PYK and PYK+NaCl

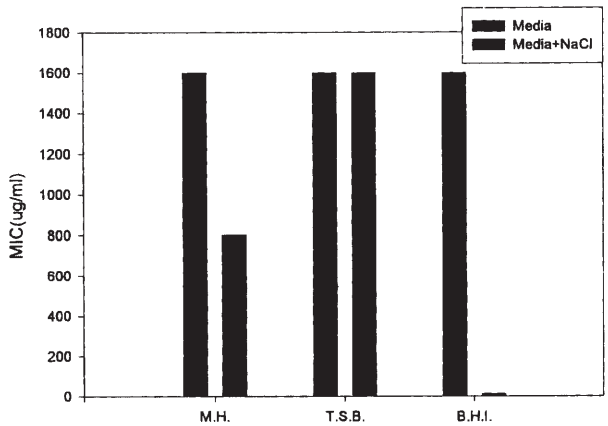

Fig. 2. Influence of NaCl on methicillin MICs in various media at 30°C. The lowering MICs at 30°C by NaCl occurred in BHI medium.

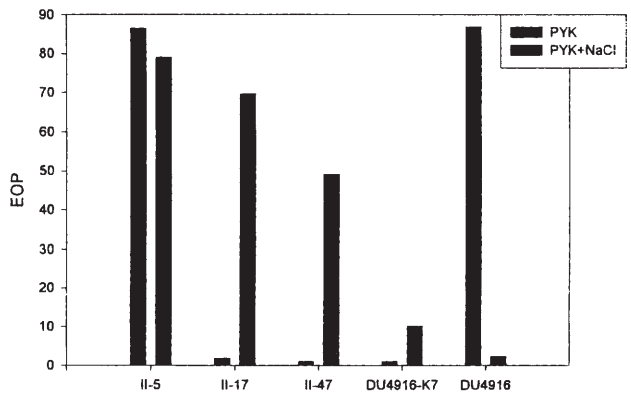

Fig. 3. Influence of NaCl on EOP at 30°C. Inclusion of NaCl increased the EOP of strain II-17, II-47 and DU4916-K7, that was opposite to the effects of NaCl compared with those of MICs at 30°C.

media. In contrast, the homogeneous methicillin resistant strain DU4916 had lower EOPs with NaCl in the medium. The reason for this is unknown. The difference in composition between liquid and solid PYK medium is the presence of agar and a complex and inert polysaccharide in the solid medium. Divalent cations (Ca^{++}, Mg^{++}) are associated with this agar, confirmed with the analysis of the composition of each media (Table 1). Divalent cations in agar medium may prevent the decrease of the methicillin resistance by NaCl, which was shown in liquid medium at 30°C.

Autolysis

To investigate the possible autolytic stimulation by NaCl which may be thought to decrease MIC in PYK+NaCl medium at 30°C, the autolytic activity of DU4916-K7 under different conditions was determined (Fig. 4). Autolysis of DU4916-K7 grown in PYK and PYK+divalent cations media were similar. And an increased autolysis was shown in PYK+NaCl and PYK+divalent cations+NaCl media. Thus, inclusion of NaCl resulted in enhanced rates of autolysis. Similar results were obtained at 40°C, but with rates of autolysis.

Penicillin binding proteins (PBPs)

All clinical isolates of methicillin-resistant *Staphylococcus aureus* contain an extra PBP2a in addition to the four PBPs present in all staphylococcal strains (3). This PBP2a is thought to be a transpeptidase essential for the continued peptidoglycan synthesis and growth in the presence of beta-lactam antibiotics. The production of PBPs under various conditions was studied. An assay of PBPs is complicated if the organism under study is a beta-lactamase producer, which destroys the radiolabeled penicillin used to label the PBPs. Hence, the strains were first assayed for beta-lactamase activity after growth in the presence or absence of methicillin. As expected, strain DU4916-K7 was negative and strain DU4916 was positive for beta-lactamase production (14). Of the S. epidermidis strains studied, II-5, II-17 and ATCC 14990 were constitutive, whereas strain II-47 was inducible. Consequently, membranes were preincubated with clavulanic acid to inhibit beta-lactamase before assaying for PBPs. First, membranes were prepared from several strains grown under various conditions and their protein profiles were examined by SDS-PAGE (Fig. 5). In some methicillin resistant strains, including DU4916-K7, it is well established that PBP 2a is present in a guantity large enough to be readily seen on Coomassie Blue stained gels with an apparent Mr of 78,000 (14). This protein can be readily seen on SDS-PAGE of membranes prepared from strain DU4916-K7 grown in PYK+NaCl medium at 30°C. A band of similar Mr was seen in strain DU 4916S, but this is believed to be a different protein since this strain does not produce PBP 2a. The band tentatively identified as PBP 2a was produced by strains II-5 and II-17 grown with methicillin, and strain DU4916 (Fig. 6). Non, or very low PBP 2a was produced by strains 3a, II-5, II-17 and II-47

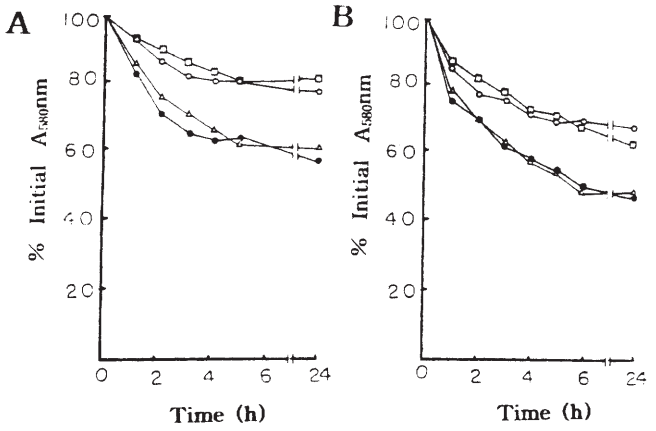

Fig. 4. Autolysis of strain DU4916 K-7 in PYK, PYK+5% NaCl, PYK+divalent cations, PYK+divalent cations+5% NaCl media at 30°C and 40°C. Media : ○, PYK; ●, PYK+NaCl; □, PYK+divalent cations; △, PYK+divalent cations+NaCl. Temperature : A, 30°C; B, 40°C.

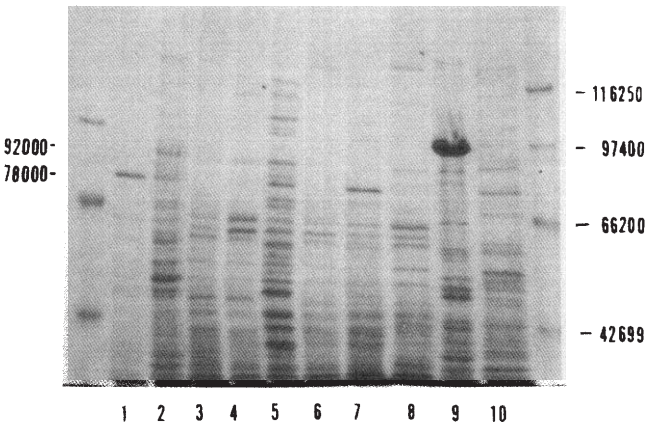

Fig. 5. Membrane protein profiles of methicillin-resistant and -susceptible strains. Membranes are isolated from the organisms grown under various conditions and were incubated with clavulanic acid followed by ^{3}H-benzylpenicillin, and membrane proteins were subjected to SDS-PAGE. The gel was stained with Coomassie blue. The fluorogram shown in Fig. 6 was also prepared with the same gel. Lanes : 1, DU 4916 K-7 (PYK+5% NaCl); 2, DU4916S (PYK+5% NaCl); 3, 3a (PYK+5% NaCl); 4, II-5 (PYK+5% NaCl); 5, II-5 (PYK+ 5 μg/ml methicillin); 6, II-17 (PYK+5% NaCl); 7, II-17 (PYK+5 μg/ml methicillin); 8, II-47 (PYK+5% NaCl); 9, II-47 (PYK+5 μg/ml methicillin); 10, DU4916 (PYK+5% NaCl).

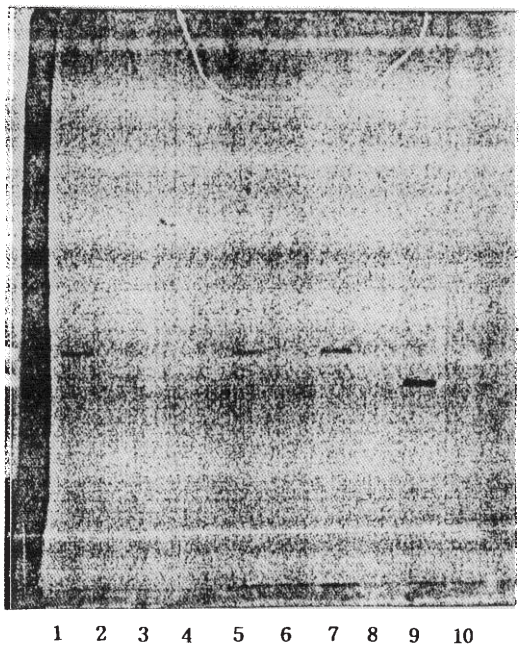

Fig. 6. Detection of penicillin-binding proteins by a fluorography. The fluorogram was prepared from the gel shown in Fig. 5. PBP2a was produced by strain DU4916-K7, II-5 and II-17 in the presence of methicillin, but not in its absence.

grown in the absence of methicillin. A massive amount of an Mr 92,000 protein was induced by growing strain II-47 in the presence of methicillin. No PBP2a was detected in strain II-47 grown with or without methicillin. However, a PBP of Mr 67,000 was detected in this strain. Thus among the methicillin-resistant *S. epidermidis* strains studied, PBP 2a production was highly dependent upon the presence of methicilllin in the medium. Similar findings were reported by Gaisford & Reynolds (6) with the *S epidermidis* strain.

Consequently, stimulation of autolysis by components of media, such as NaCl, peptone may be responsible for reducing the expression of methicillin resistance. Divalent cations have crucial roles in bacterial cell walls and membranes (4). Presence of divalent cations in media can stabilize the bacteria damaged with NaCl or peptone; and this stabilization improves the chance of survival of bacteria in the presence of methicillin by production of PBP2a, which takes over the roles of other saturated PBPs.

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