ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 2008, p. 3905–3908 Vol. 52, No. 11
0066-4804/08/$08.00+0 doi:10.1128/AAC.00653-08

In Vitro and In Vivo Evaluations of Oxacillin Efficiency against *mecA*-Positive Oxacillin-Susceptible *Staphylococcus aureus*▽

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Received 17 May 2008/Returned for modification 21 June 2008/Accepted 5 August 2008

Community-type *Staphylococcus aureus* strains that are positive for *mecA* and PBP2a but appear phenotypically susceptible to oxacillin are increasingly reported worldwide. Four *S. aureus* clinical isolates carrying the *mecA* gene with oxacillin MICs of <2 μg/ml were tested for oxacillin efficiency by population analyses and experimental thigh infections. These isolates harbored staphylococcal cassette chromosome *mec* type IV and belonged to two genotypes. Two of the four isolates were found by population analysis to be truly oxacillin susceptible. All four isolates exhibited significant reductions in the numbers of colonies grown after dicloxacillin treatment of experimental thigh infections, as also did a *mecA*-negative *S. aureus* control strain. These observations indicate that some of the phenotypically oxacillin susceptible *mecA*-positive *Staphylococcus aureus* isolates may be at least partially responsive to oxacillin.

Staphylococcus aureus isolates that either have oxacillin MICs higher than 2 μg/ml or harbor the *mecA* gene are considered methicillin resistant (MRSA) by the Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) (12). Oxacillin resistance in *mecA*-positive isolates is due to the production of an altered form of penicillin-binding protein (PBP), designated PBP2a, which shows low affinity for beta-lactams (2). *S. aureus* strains that carry and express the *mecA* gene may exhibit oxacillin MICs ranging from <2 to >1,000 μg/ml and resistance phenotypes ranging from extremely heterogeneous (frequencies of highly resistant colonies, 10^{-7} or lower) to homogeneous (7, 13, 18).

It is generally believed that most *mecA*-positive, oxacillin-susceptible *S. aureus* strains exhibit oxacillin heteroresistance, even at a low frequency, and that the use of beta-lactams might lead to treatment failure (14). However, it is believed that chromosomal factors unrelated to the *mecA* gene, which confer different levels of the oxacillin-heterogeneous phenotype, should exist but remain unknown and are probably pleiotropic, complex, and strain dependent (13). In this context, one could anticipate that a proportion of nonheterogeneous, functionally oxacillin susceptible, *mecA*-positive *S. aureus* (OS-MRSA) strains also exist. The treatment alternatives for such isolates would thus be broadened, possibly including penicillinase-resistant beta-lactams. The aim of this study was to investigate by in vitro and in vivo experiments the characteristics of OS-MRSA strains and the possible efficiency of oxacillin against them, in a region where the rate of *mecA*-positive *S. aureus* is among the highest in Europe (4) and community-type OS-MRSA strains are increasingly isolated.

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▽ Published ahead of print on 11 August 2008.

MATERIALS AND METHODS

Bacterial strains and susceptibility testing. Four *mecA* gene-carrying *S. aureus* clinical isolates (SA 1306, SA 1326, SA 1552, and SA 4666) that were phenotypically oxacillin susceptible (oxacillin MIC, ≤1 μg/ml) and were collected from October to November 2006 were studied. A low-level (SA 6083; oxacillin MIC, 6 μg/ml; *mecA* positive) and a high-level (SA 2712; oxacillin MIC, 256 μg/ml; *mecA* positive) MRSA clinical isolate from our laboratory collection, as well as *S. aureus* ATCC strain 29213 (methicillin susceptible [MSSA], *mecA* negative), were included for comparison. Species identification was performed with the Vitek 2 system (bioMerieux, Marcy l'Etoile, France) using an ID-GP card, and the initial antimicrobial susceptibility status was determined with a GPS-105 card. The oxacillin MIC was estimated by Etest (AB Biodisk, Solna, Sweden) in Mueller-Hinton agar plates containing 2% NaCl and also by the agar dilution method according to CLSI guidelines (12) using 2-μg/ml increments for concentrations of 2 to 16 μg/ml. Susceptibility to other beta-lactams (ampicillin, amoxicillin-clavulanate, cefamandole, cephalexin, and imipenem) was tested by Etest.

Detection of PBP2a and the *mecA* gene, and typing assays. The Slidex MRSA latex agglutination test (bioMerieux) was performed to detect PBP2a. A PCR specific for the *mecA* gene, sequencing of *mecA*, and PCR typing of the staphylococcal cassette chromosome *mec* (SCC*mec*) element were carried out with previously described primers and amplification conditions (9, 11). Pulsed-field gel electrophoresis (PFGE) of SmaI-digested genomic DNA was performed as previously described (19), and bands were compared visually (17). Multilocus sequence typing (MLST) was also performed (3). Sequences of both strands were determined at Lark Technologies, Inc. (Takeley, United Kingdom) and analyzed using DNAStar (Madison, WI) software (Lasergene, version 5.07); MLST types were assigned through the MLST database (http://www.mlst.net).

Population analysis assays. Overnight broth cultures were diluted in cation-adjusted Mueller-Hinton broth to a density equivalent to a 4 McFarland standard (approximately 10^9 CFU). From this starting suspension, 100 μl (approximately 10^8 CFU), 10 μl (approximately 10^7 CFU), and 1 μl (approximately 10^6 CFU) were spread onto Mueller-Hinton agar plates containing dicloxacillin (sodium dicloxacillin monohydrate, Diclocil; Bristol-Myers Squibb) according to the protocol originally described by Tomasz et al. (18). A lower inoculum of approximately 10^4 CFU was spread for isolates that displayed confluent growth with the starting inocula given above. Dicloxacillin was incorporated into the plates in serial twofold dilutions for concentrations of 0.25 to 128 μg/ml, with 1-μg/ml increments from 1 to 6 μg/ml and 2-μg/ml increments from 6 to 16 μg/ml, in order to detect more precisely the changes in the susceptibilities of the heteroresistant populations. Colonies were counted after 48 h of growth at 35°C. The analysis was performed three times for all isolates, and the median CFU grown in each concentration was counted and plotted on a semilogarithmic graph. The population analysis protocol (18) was also performed after addition of 2% NaCl to the medium. Population analyses of the tested isolates were compared with those of the low- and high-level MRSA isolates and ATCC 29213.

TABLE 1. Characteristics of study isolates and data for experimental thigh infections

Isolate	Date of isolation (mo/day/yr)	Ward[a]	Specimen source	PFGE type	ST	SCC*mec* type	Oxacillin agar dilution MIC (μg/ml)	Highest oxacillin concn (μg/ml) at which cell growth occurred[b]	Avg log CFU ± SD (%) per g thigh tissue			Susceptibility status defined by Vitek 2[c]	
									Untreated controls	Treated animals	*P* (treated vs untreated animals)	OXA	VAN
1306	10/2/06	MED	Sputum	Ia	728	IV	0.5	32	6.55 (8.6)	4.71 (9.7)	<0.001	R	S
1326	10/2/06	DERM	Urine	Ib	728	IV	0.25	0.5	6.6 (6.5)	4.50 (4.3)	<0.001	S	S
1552	10/4/06	HAEM	Pus	II	728	IV	1	64	6.25 (10.3)	3.75 (9.1)	<0.001	R	S
4666	11/1/06	DERM	Pus	Ic	728	IV	1	1	6.53 (8.3)	3.72 (10.4)	<0.001	S	S
6083	11/14/06	OBST	Skin	Ic	728	IV	6	128	7.45 (11.2)	5.02 (6.2)	<0.001	R	S
2712	6/22/07	Surgery	Blood	III	ND[d]	ND	256	>128	6.32 (3.6)	6.25 (8.8)	NS[e]	R	S
29213	NA[f]	ATCC	NA	IV	ND	NA	0.125	0.5	6.70 (6.7)	1.18 (12.4)	<0.001	S	S

[a] MED, medicine; DERM, dermatology; HAEM, hematology.
[b] By population analysis.
[c] OXA, oxacillin; VAN, vancomycin; S, susceptible; R, resistant.
[d] ND, not determined.
[e] NS, not significant.
[f] NA, not applicable.

To determine the molecular stability of the *mecA* genotype, the colonies grown in the highest dicloxacillin concentration were tested by PCR for the presence of the *mecA* gene and also for PBP2a production, as described above. Because most clinicians treating infections due to *mecA*-positive *S. aureus* will be utilizing vancomycin, population analysis was also performed for vancomycin in parallel with dicloxacillin.

Oxacillin bactericidal assays. Overnight Mueller-Hinton broth cultures were diluted with cation-adjusted Mueller-Hinton broth, and approximately 10^6 to 10^7 CFU was inoculated into Mueller-Hinton broth containing 20 μg/ml dicloxacillin (14). Viable cells were counted by spreading 0.1-ml aliquots of the broth cultures at 0, 1, 3, 6, 9, 14, 24, 30, 42, and 48 h of incubation at 35°C. The analysis was performed three times for all isolates, and the median CFU grown in each concentration was counted and plotted on a semilogarithmic graph. A bactericidal assay was also performed for vancomycin at 10 μg/ml, as described previously (15).

Mouse thigh infection model. Male Wistar rats (body weight, 300 to 400 g) were utilized for experimental thigh infections following a previously described protocol (6). In brief, a starting inoculum of approximately 10^8 CFU/ml was prepared in Mueller-Hinton broth, and 100 μl was injected into the left thigh of the animal. Sterile water was injected into the right thigh. Two hours after the bacterial inoculation and every 6 h thereafter, dicloxacillin at 200 mg/kg of body weight was administered subcutaneously. The animal was sacrificed at 24 h; the thigh muscle was homogenized; and the number of viable CFU was counted after serial dilution of the homogenate. The infections were carried out in triplicate for the four *mecA*-positive, oxacillin-susceptible isolates as well as for the control strains ATCC 29213, SA 6083, and SA 2712. Also, triplicate control experiments were performed with oxacillin-susceptible, *mecA*-positive isolates that were injected in the left thigh but not treated with dicloxacillin. The colonies grown from the infected thighs were tested for the presence of the *mecA* gene and for production of PBP2a.

Statistical analysis. A *t* test of mean CFU values obtained in the mouse thigh infection model was performed using GraphPad Prism software, version 4.00 (GraphPad Software, Inc.). Data for which the *P* value was <0.05 were considered statistically significant.

RESULTS

The oxacillin MICs of the four study isolates by both Etest and agar dilution ranged from 0.25 to 1 μg/ml. Two of the four isolates were also classified as susceptible to oxacillin by the Vitek 2 system. All isolates were susceptible to vancomycin, teicoplanin, linezolid, and quinupristin-dalfopristin. Two isolates were also susceptible to clindamycin, erythromycin, and fusidic acid, and one was also susceptible to tetracycline. All four isolates were also found by Etest to be susceptible to ampicillin, amoxicillin-clavulanate, cefamandole, cephalexin, and imipenem. A PBP2a latex agglutination test was positive for the four study isolates. PCR was positive for the *mecA* gene, and its nucleotide sequence in the four isolates was identical to that previously described for several MRSA strains, including *S. aureus* COL (5; GenBank accession number CP000046). The four isolates belonged to two unrelated genotypes, of which the more common strain pattern, which also included the control strain SA 6083, exhibited three subtypes differing from each other by one to two bands (Table 1). It should be noted that PFGE profile I (Table 1) was similar to the PFGE profile of a previously reported strain from southwestern Greece (type C, sequence type 80 [ST-80] [1]). MLST results showed that the four isolates of PFGE type I belong to ST-728 (allelic profile, 1-3-1-14-11-27-10), which is a single-band variant of ST-80. All isolates carried *SCCmec* type IV, as shown by PCR typing.

Population analysis showed that two of the isolates (SA 1326 and SA 4666) (Table 1) were phenotypically oxacillin susceptible: the highest oxacillin concentrations at which cell growth occurred were 0.5 and 1 μg/ml, respectively. The remaining two isolates (SA 1306 and SA 1552) grew at oxacillin concentrations as high as 32 and 64 μg/ml, respectively. The low-level-resistant isolate, SA 6083, grew at oxacillin concentrations as high as 128 μg/ml, while the populations of the high-level-resistant isolate, SA 2712, remained unaffected up to 128 μg/ml (Fig. 1A). After the addition of 2% NaCl to the medium, the oxacillin-susceptible isolates SA 1326 and SA 4666 grew at oxacillin concentrations as high as 1 and 2 μg/ml, respectively. The colonies grown in the highest dicloxacillin concentration in the population analysis still carried and expressed the *mecA* gene in all isolates, including the nonheterogeneous isolates SA 1326 and SA 4666, suggesting the stability of the *mecA* genotype. Time-kill kinetics showed that at 24 h, oxacillin had bactericidal activity for the oxacillin-susceptible isolates (SA 1306, SA 1326, SA 1552, SA 4666, and ATCC 29213) as well as for the low-level-resistant control, SA 6083, while the high-level oxacillin-resistant control, SA 2712, remained unaffected (Fig. 2). All isolates were susceptible to narrow-spectrum vancomycin in population analyses (Fig. 1B), and time-kill curves

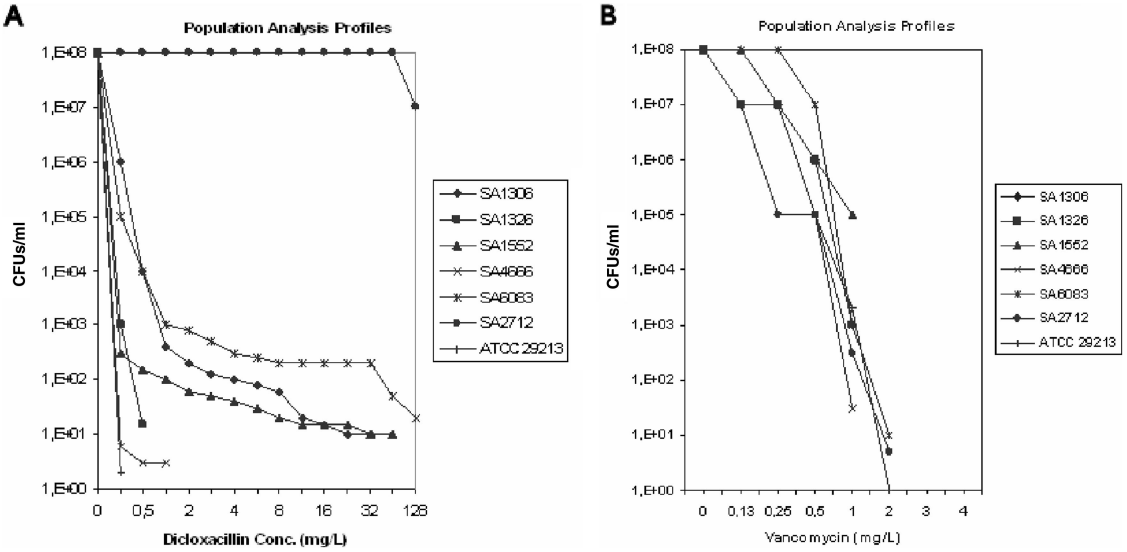

FIG. 1. Population analysis profiles of the study isolates for dicloxacillin (A) and vancomycin (B). Each symbol represents the mean for three replicates.

showed vancomycin bactericidal activity at a level similar to that reported previously for community-associated MRSA (15).

Testing of oxacillin efficiency in the experimental mouse thigh model showed no significant differences in the number of colonies yielded 24 h after inoculation between the four *mecA*-positive, oxacillin-susceptible isolates and the oxacillin-susceptible, *mecA*-negative isolate (ATCC 29213) (Table 1). For all four study isolates, statistically significantly fewer colonies were obtained from the dicloxacillin-treated animals than from their respective untreated controls ($P < 0.001$). The colonies yielded from all infected thighs also carried and expressed the *mecA* gene and had susceptibility profiles similar to those of the respective clinical isolates.

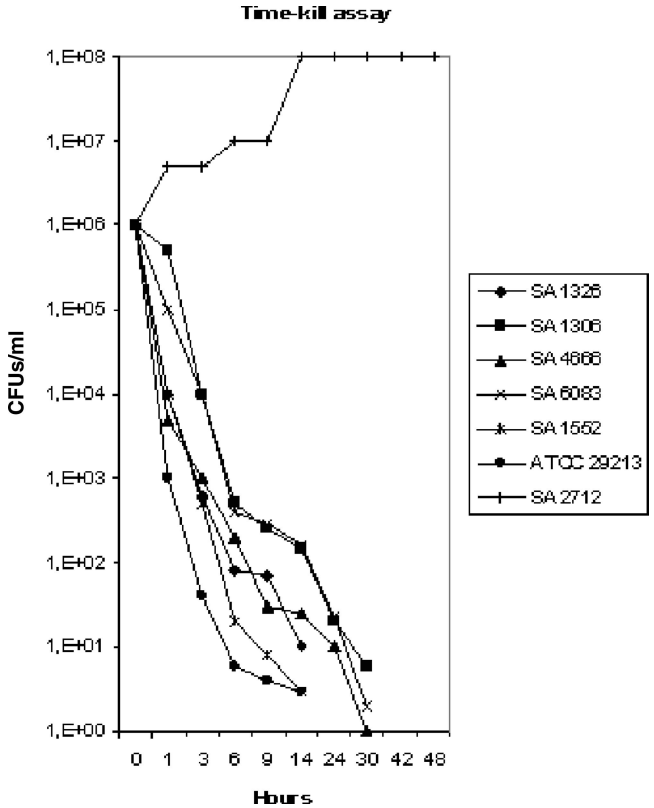

FIG. 2. Oxacillin bactericidal assay of the study isolates in Mueller-Hinton broth with 20 μg/ml dicloxacillin. Each symbol represents the mean for three replicates.

DISCUSSION

S. aureus clinical isolates that carry the *mecA* gene but appear phenotypically oxacillin susceptible have been increasingly reported recently (7, 14). It has been suggested that such isolates could be classified as a new type of MRSA, designated OS-MRSA, which may be misclassified as MSSA in the daily routine if only susceptibility to antimicrobials is tested (7). It is generally believed that when treating OS-MRSA infections, we should take precautions, because treatment with beta-lactam antibiotics may result in the emergence of highly resistant MRSA, which is attributable to the presence of the *mecA* gene (14). However, new types of hetero-MRSA strains that are phenotypically susceptible to narrow-spectrum cephalosporins and imipenem have recently been observed in hospitals (10). In this study, we investigated *S. aureus* clinical isolates that were *mecA* positive but exhibited oxacillin MICs of ≤1 μg/ml and were susceptible to various beta-lactams. Two of the study isolates were misclassified as MSSA by the very sensitive Vitek 2 system (14). During population analysis, these isolates grew at low oxacillin concentrations.

The observations of the present study raise the need for identification of additional putative genetic factors that could play an important role in this degree of heteroresistance. It is evident that any factor playing a role in cell wall synthesis and assembly could represent such a putative factor. For example, the profound disturbance of cell wall metabolism associated

with vancomycin resistance has been correlated previously with suppression of the methicillin resistance level in those bacteria ("seesaw" effect) (16). Furthermore, since oxacillin resistance levels depend strongly on environmental conditions, specific global regulatory networks should exist and play a role in heterogeneous resistance, and their activation or suppression would lead to the pleiotropic effects that allow for "susceptible" or highly resistant subclones (13). Also, alterations in the activity of autolysins, which have been shown to affect the level of methicillin resistance, should be investigated more closely (13). A recent report (8) showed that deletion of the important (but not essential for viability) FemA and FemB factors, which are involved in cell wall synthesis, could lead to a global transcriptional rearrangement in *S. aureus* involving oxacillin responsiveness. In this context, DNA array analysis showed that specific genes whose expression was down- or upregulated in FemA-FemB deletion mutant strains represent major metabolic pathways that have, so far, been considered uncoupled. Finally, it is possible that random gene rearrangements through transposons or insertion sequences could lead to stable genetic alterations that could contribute to heterogeneous expression of oxacillin resistance.

Whatever the underlying reasons, it is evident from our report that although *mecA* detection and expression are prerequisites for methicillin resistance, such a genotype does not guarantee phenotypic methicillin resistance. The effort to elucidate the causes of heteroresistance should be expanded to the detection and characterization of new molecular targets that could be associated with the atypical susceptibility that many nosocomial *S. aureus* strains already exhibit. Elucidation of the origins of heteroresistance to methicillin may also contribute to the discovery of new targets for antibiotics (13).

From the results of the present study, the response of the study isolates to oxacillin apparently lies between that of a fully susceptible strain (ATCC 29213) and that of a highly resistant MRSA strain. Since more "susceptible" MRSA clones are gradually emerging worldwide, investigation of the origins of heteroresistance and of a putative degree of activity for oxacillin, compared with vancomycin, against OS-MRSA strains, may provide some insights on possible therapeutic alternatives. However, based on the current knowledge that some strains become more homogeneously oxacillin resistant when exposed to oxacillin in vitro and in vivo, additional animal models are clearly needed in order for similar assumptions to be substantiated. In this context, experiments involving larger collections of isolates and direct comparisons of oxacillin versus vancomycin efficiency are currently in progress.

When we compared previously reported OS-MRSA appearances in other regions (7, 14), we noticed that such isolates are quite common in our region. Particularly, during the 1-year period from August 2006 to July 2007, 26 of 167 (15.5%) *mecA*-positive strains were OS-MRSA (oxacillin Etest MICs in 2% NaCl, <2 μg/ml), while from August 2007 to April 2008, the respective proportion dropped to 11.8% (22 of 187). In this context, we are currently screening all *S. aureus* isolates with oxacillin MICs ranging from 0.25 to 16 μg/ml for PBP2a production, in order to annotate OS-MRSA strains correctly. In addition, we are currently investigating genetic factors that could affect the expression of the oxacillin resistance phenotype in such strains.

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