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Borderline Methicillin-Susceptible *Staphylococcus aureus* Strains Have More in Common than Reduced Susceptibility to Penicillinase-Resistant Penicillins

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Ten epidemiologically unrelated *Staphylococcus aureus* isolates with borderline levels of susceptibility to antistaphylococcal penicillinase-resistant penicillins (PRPs) were investigated together with appropriate *S. aureus* control strains. By a nitrocefin microplate assay, all borderline PRP-susceptible test strains were found to produce comparable amounts of β-lactamase. Hydrolytic activity against another chromogenic substrate (PADAC) and against the PRPs was also demonstrated in membrane preparations from induced cells of 9 of the 10 borderline test strains. When bacterial membranes were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, two methicillin-inducible bands of about 32 and 31 kDa were detected, after Coomassie blue staining, in the membrane protein patterns of the same nine borderline test strains. By gel renaturation and zymographic detection of β-lactamase activity, both methicillin-inducible membrane proteins were detected with nitrocefin as a substrate, whereas only one band (presumably the smaller protein) was detected with PADAC. With the remaining borderline test strain (a40), only the larger band was detected in the renatured gels with nitrocefin as a substrate. Plasmid DNA analysis revealed that the borderline susceptible test strains, again with the exception of a40, shared a 17.2-kb plasmid yielding four *Hin*dIII fragments of 7.0, 5.3, 3.5, and 1.4 kb. In Western blot (immunoblot) experiments using rabbit antiserum to penicillin-binding protein (PBP) 2a, test strain a40, which did not share a number of features characteristically associated with the other borderline test strains, was eventually shown to produce PBP 2a. Five other *S. aureus* strains, belonging to phage group 94/96, were found to display the borderline phenotype, including such distinguishing features as the membrane-associated PRP- and PADAC-hydrolyzing activity, the two methicillin-inducible membrane proteins, and the 17.2-kb plasmid. These results suggest that borderline susceptible *S. aureus* strains share more common features than reduced susceptibility to PRPs.

Over the past few years, special attention has been focused on *Staphylococcus aureus* strains with borderline levels of susceptibility or resistance to methicillin and related antistaphylococcal penicillinase-resistant penicillins (PRPs) (30). Thornsberry and McDougal first reported the occurrence of *S. aureus* strains with intermediate susceptibility to PRPs (17, 27) and then defined the concept of borderline susceptibility (or low-level resistance) by postulating that in these *S. aureus* strains—which were neither heteroresistant nor multidrug resistant, produced large amounts of β-lactamase, and became fully susceptible to PRPs in the presence of β-lactamase inhibitors—the borderline MICs were due to hyperproduction of β-lactamase (18). The nonheterogeneous expression of this reduced susceptibility to PRPs was confirmed by population analysis (20, 31). Its nonintrinsic nature was confirmed by the experimental findings that borderline susceptible *S. aureus* strains did not contain DNA that hybridized with probes specific for the methicillin resistance determinant (*mec*) (4) and did not produce the low-affinity penicillin-binding protein (PBP) 2a (4, 20). It was argued that there is no apparent reason for borderline *S. aureus* isolates to be subject to the current practice of considering methicillin-resistant staphylococci as resistant to all other β-lactams (20) and that infections caused by these strains can probably be safely and effectively treated with β-lactam antibiotics (4).

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However, the matter is probably more complex than originally believed. In particular, there is increasing evidence that the borderline phenotype cannot be defined solely on the basis of β-lactamase hyperproduction: in fact, both borderline susceptible *S. aureus* strains which do not hyperproduce β-lactamase and fully PRP-susceptible strains which do hyperproduce it have been reported (2, 5, 15, 26, 32). Moreover, mechanisms involving PBP modification may also lead to reduced staphylococcal susceptibility to PRPs. One such mechanism is characterized by the presence of the *mec* gene and PBP 2a (5, 28) and is similar, except for the lower PRP MICs, to conventional high-level resistance. Another mechanism, best documented in homogeneous, β-lactamase-negative strains (3, 28, 29), is associated with altered binding capacity of normal PBPs (i.e., in the absence of PBP 2a) possibly resulting from point mutations in the PBP 2 gene (7).

We have recently shown that, in addition to the classical penicillinase, borderline PRP-susceptible *S. aureus* strains produce a second, methicillin-hydrolyzing β-lactamase (methicillinase) having a molecular mass slightly smaller than that of the penicillinase (15). By plasmid DNA analysis, we subsequently noted that the methicillinase-producing strains possessed a common 17.2-kb β-lactamase plasmid (14) apparently identical to a penicillinase-encoding plasmid described in borderline PRP-susceptible *S. aureus* isolates involved in widespread nosocomial infections (19). Moreover, a close correlation between the borderline phenotype and phage group 94/96 has been reported in studies concerning *S. aureus* strains associated with nosocomial infections (19, 34). In the present study, these

features associated with borderline *S. aureus* strains have been further investigated with the purpose of better characterizing these organisms and providing more reliable markers to define them.

MATERIALS AND METHODS

Bacterial strains. Ten *S. aureus* strains (a3, a15, a28, a40, a56, a82, a217, a263, a404, and a577)—independently isolated from a variety of clinical specimens and representing geographic and chronologic diversities—were selected on the basis of borderline PRP susceptibility levels (MIC of methicillin, 4 to 8 μg/ml; MIC of oxacillin, ≤2 μg/ml); PRP MICs proved to be reproducible in repeated assays. Five additional *S. aureus* strains belonging to phage group 94/96 (the propagating strains for the basic-set typing phages 94 and 96 and three unrelated strains examined for routine phage typing) were obtained from the phage typing reference laboratory in Italy (National Institute of Health, Rome, Italy). Two *S. aureus* control strains from our institute's collection included a53 (a borderline isolate previously characterized for PBPs [20], β-lactamase activity [14, 15], and plasmid DNA [14]) and a90 (a fully PRP-susceptible isolate with high-level resistance to penicillin G). *S. aureus* ATCC 25923 and ATCC 29213 were used as β-lactamase-negative and -positive controls, respectively.

Penicillins and β-lactamase substrates. Penicillin G, methicillin, oxacillin, cloxacillin, dicloxacillin, and nafcillin were purchased from Sigma Chemical Co., St. Louis, Mo. The chromogenic cephalosporins nitrocefin and PADAC were purchased from Oxoid Ltd., Basingstoke, United Kingdom, and Calbiochem, San Diego, Calif., respectively.

Susceptibility tests. MICs were determined by the broth microdilution method as recommended by the National Committee for Clinical Laboratory Standards (21). As recommended in particular for PRP susceptibility tests, the inoculum was prepared directly from an overnight agar plate, and 2% NaCl was added to the cation-adjusted Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.). Penicillins were tested at final concentrations (prepared from serial twofold dilutions) ranging from 0.125 to 128 μg/ml for penicillin G and from 0.25 to 32 μg/ml for methicillin and oxacillin. *S. aureus* ATCC 29213 was used for quality control.

Preparation of membrane fraction. Bacteria were grown to the mid-logarithmic phase in tryptic soy broth (Difco) at 37°C with shaking. The medium was used unsupplemented or supplemented with methicillin (0.5 μg/ml) as a β-lactamase inducer. The cultures were harvested after 3 h of exposure to the inducer. Bacterial membranes were prepared as previously described (15) and resuspended in 10 mM phosphate buffer (pH 7.0) at a concentration of 2 mg/ml. The protein content was determined with a DC protein assay kit (Bio-Rad Laboratories, Richmond, Calif.).

β-Lactamase assays. A nitrocefin microplate assay was similar to the method originally applied by McDougal and Thornsberry (18). Aliquots of 50 μl of a 500-μg/ml nitrocefin solution were added to two selected wells of broth microdilution plates grown overnight: the growth control well (to test uninduced β-lactamase) and the well containing 0.5 μg of methicillin per ml (to test induced β-lactamase). On the basis of color change and reaction time, the results were scored as −, 1+, 2+, or 3+ according to a previously described scheme (15, 32).

β-Lactamase in the membrane fraction, prepared as described above, was tested by spotting 20 μg of membrane protein on 1% agarose plates as reported previously (14). Briefly, with the PRPs or penicillin G used as a substrate (1%, wt/vol), hydrolytic activity was tested by an iodometric technique and revealed by the appearance of a zone of clearing in the dark background. With the chromogenic substrates (100 μM), hydrolytic activity was directly indicated by color change (red for nitrocefin or yellow for PADAC). A crude extract of *Escherichia coli* C600(R46), which produces OXA-2 β-lactamase (10), was prepared as described elsewhere (14) and used as a positive control for all substrates.

Analysis of membrane proteins by SDS-PAGE and detection of β-lactamase activity by gel renaturation. Bacterial membranes (aliquots of 50 to 100 μg of total protein) were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The stacking and separating gels were 5 and 15% polyacrylamide at pHs 6.8 and 8.8, respectively. The gels were run at 10 mA for 24 h in a continuous SDS-Tris-glycine buffering system. After electrophoresis, the gels were either stained with Coomassie brilliant blue R250 or renatured in phosphate buffer containing 1% (vol/vol) deionized Triton X-100 (16). In the latter case, after 6 h of incubation, necessary to renature staphylococcal β-lactamases (15), the gels were overlaid with 1% agarose gel containing nitrocefin or PADAC (100 μM). Hydrolytic activity was detected after incubation up to 30 min at room temperature when nitrocefin was used as a substrate and overnight at 37°C when PADAC was used.

Preparation of plasmid DNA and agarose gel electrophoresis. Plasmid DNA was isolated with a Wizard Minipreps DNA Purification System (Promega Corp., Madison, Wis.). Lysostaphin (Sigma) was added to the cell resuspension buffer at a final concentration of 0.1 μg/ml. Plasmid DNA was digested with *Hin*dIII (Boehringer Mannheim, Mannheim, Germany), and restriction fragments were separated by electrophoresis on horizontal 0.8% agarose gels with TAE buffer (40 mM Tris acetate [pH 8.5], 2 mM EDTA) and stained with ethidium bromide (0.5 μg/ml).

Immunodetection of PBP 2a. PBP 2a was obtained from *S. aureus* 27R (methicillin resistant, producing PBP 2a constitutively [9, 29]). After separation of membrane proteins by SDS-PAGE and staining with Coomassie blue, a slice of the gel containing PBP 2a was taken, washed, homogenized, and used to immunize New Zealand White rabbits (8). The antiserum was stored at −20°C in the presence of 0.02% azide and, before use in immunoblot experiments, preadsorbed to membranes from *S. aureus* 27S (methicillin susceptible, isogenic with 27R [9, 29]). Samples of membrane fraction (150 μg of total protein) were prepared from *S. aureus* cells grown at 30°C in tryptic soy broth containing 4% NaCl and separated by SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, Mass.) in a semidry system (Millipore) for 1 h at 0.8 mA/cm^2 (8) and immunoblotted with anti-PBP 2a antiserum (1:2,500). After washing, a second antibody consisting of alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Boehringer Mannheim) was applied. This was followed by extensive washing and visualization with an alkaline phosphatase substrate kit (Bio-Rad).

RESULTS

β-Lactamase activity of borderline *S. aureus* strains. The 10 borderline-PRP-susceptible *S. aureus* test strains were assayed for β-lactamase activity with different substrates. *S. aureus* a53 (previously shown to produce an inducible methicillinase activity [15]), a90, ATCC 29213, and ATCC 25923 were tested as controls. β-Lactamase activity was scored from uninduced and induced cultures by the microplate nitrocefin assay. By this method, all of the borderline test strains appeared to produce large amounts of β-lactamase, particularly after methicillin induction. Hydrolytic activity against nitrocefin, PADAC, penicillin G, and different PRPs (methicillin, oxacillin, cloxacillin, dicloxacillin, and nafcillin) was then tested by agarose plate assays from cytoplasmic membranes prepared from uninduced and induced cells. No activity on any substrate was shown by the β-lactamase-negative control (ATCC 25923), whereas nitrocefin and penicillin G were hydrolyzed by all other 13 *S. aureus* strains. Of the latter, 10 (a53 and 9 of the 10 test strains) also hydrolyzed the PRPs and PADAC, provided that membrane preparations from induced cells were used. The remaining borderline test strain (a40) and the fully PRP-susceptible controls a90 and ATCC 29213 did not hydrolyze either the PRPs or PADAC under any condition. A summary of the susceptibility and β-lactamase activity data is reported in Table 1. The results obtained with membrane fractions from induced cells with nitrocefin, PADAC, penicillin G, methicillin, and oxacillin as substrates are shown in Fig. 1.

Analysis of membrane proteins by SDS-PAGE. For each strain, cytoplasmic membrane proteins obtained from cells grown both in the absence and in the presence of methicillin (0.5 μg/ml) were separated by SDS-PAGE and stained with Coomassie blue. Two closely migrating but distinct methicillin-inducible bands, with molecular masses of about 32 and 31 kDa, were detected in the membrane fractions of 9 of the 10 borderline-susceptible test strains. The results obtained with three such strains (a3, a28, and a56) are shown in Fig. 2. The two bands appeared to be the same as those detected in the membrane fraction of the borderline *S. aureus* strain a53 (Fig. 2, lane 12) and previously shown to possess β-lactamase activity (15). With the remaining borderline test strain (a40) and control strains a90 and ATCC 29213, as well as with the β-lactamase-negative control ATCC 25923, such methicillin-inducible bands were not observed in the Coomassie blue-stained gels, even after induction (a40 and a90 are shown in Fig. 2).

Renaturation assays and zymographic detection of β-lactamase activity. A set of similar gels with cytoplasmic membrane proteins were run in parallel, renatured with Triton X-100, and assayed for β-lactamase activity. When nitrocefin was used as a substrate, the 32-kDa methicillin-inducible band—previously identified as the classical staphylococcal penicillinase (15)—was detected in all strains except the β-lactamase-negative control ATCC 25923. The second band—i.e., the 31-kDa pro-

TABLE 1. Susceptibility to penicillin G, methicillin, and oxacillin and β-lactamase activity in 14 *S. aureus* strains

Strain	MIC (μg/ml) of:			β-Lactamase activity				
				By nitrocefin microplate assay		Inducible in the membrane fraction against:		
	Penicillin G	Methicillin	Oxacillin	Uninduced	Induced	Penicillin G	PRPs	PADAC
ATCC 29213	0.5	2	0.25	1+	2+	Yes	No	No
ATCC 25923	≤0.125	2	≤0.125	−	−	No	No	No
a90	64	2	≤0.125	2+	3+	Yes	No	No
a53	128	4	2	2+	3+	Yes	Yes	Yes
a28	128	4	2	2+	3+	Yes	Yes	Yes
a3	64	4	2	2+	3+	Yes	Yes	Yes
a56	128	4	2	2+	3+	Yes	Yes	Yes
a577	>128	4	1	2+	3+	Yes	Yes	Yes
a404	128	4	2	1+	3+	Yes	Yes	Yes
a217	>128	4	2	2+	3+	Yes	Yes	Yes
a82	64	4	2	2+	3+	Yes	Yes	Yes
a15	128	4	1	2+	3+	Yes	Yes	Yes
a263	128	4	2	1+	3+	Yes	Yes	Yes
a40	64	8	0.5	2+	3+	Yes	No	No

tein previously shown to have hydrolytic activity against methicillin (15)—was detected in the same borderline strains showing this protein in the Coomassie blue-stained gels, provided that the membranes were obtained from methicillin-induced cultures. Even after induction, this second band was not detectable in the membrane fraction of either the remaining borderline test strain (a40) or control strains a90, ATCC 29213, and ATCC 25923. When PADAC was used, a band denoting hydrolysis (presumably the 31-kDa protein) was detected only in the membranes from methicillin-induced cultures of the same borderline strains showing both the 32- and the 31-kDa bands, under the same induction conditions, with nitrocefin as a substrate. The results obtained with a90 and four borderline test strains (a3, a28, a40, and a56) are shown in Fig. 3A (nitrocefin) and B (PADAC).

Plasmid DNA analysis. As well as the control strain a53, the borderline PRP-susceptible test strains (again with the exception of a40) showed the presence of a common 17.2-kb plasmid with the same *Hin*dIII restriction pattern (four fragments of 7.0, 5.3, 3.5, and 1.4 kb). Different plasmid patterns were shown by the borderline test strain a40 and the other control strains (data not shown).

Immunodetection of PBP 2a. Further studies of test strain a40 showed the presence of a 78-kDa band corresponding to PBP 2a as in the control strain 27R but not in all the other borderline susceptible test strains (data not shown).

Relationships to phage typing. The phage typing reference laboratory in Italy stopped phage typing with staphylococci in 1994 but provided us with five *S. aureus* strains previously assigned to phage group 94/96. These five strains were all found to display the borderline phenotype, as previously suggested (19, 34), with identical methicillin MICs (4 μg/ml) and oxacillin MICs ranging from 0.5 to 2 μg/ml. All showed strong β-lactamase activity, particularly after induction, and all shared additional distinguishing features, including membrane-associated PRP- and PADAC-hydrolyzing activities, the two close methicillin-inducible membrane proteins (32 and 31 kDa), and the 17.2-kb plasmid yielding the characteristic four-fragment pattern after digestion with *Hin*dIII.

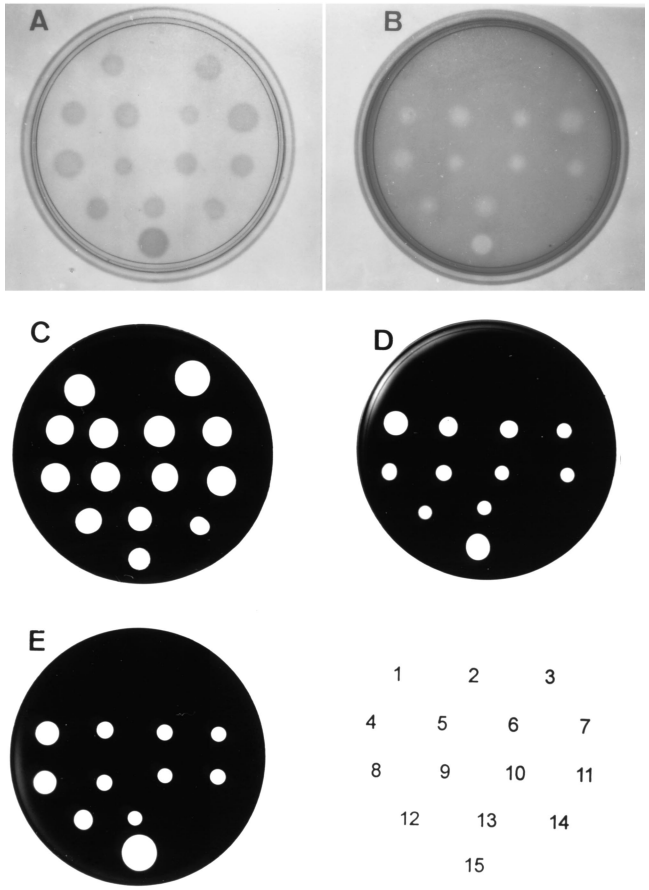

FIG. 1. β-Lactamase activity in cytoplasmic membranes from methicillin-induced *S. aureus* cells against nitrocefin (A), PADAC (B), penicillin G (C), methicillin (D), and oxacillin (E). With the chromogenic substrates nitrocefin and PADAC, hydrolytic activity was directly indicated by color change. With the other substrates, the hydrolytic activity was assayed by an iodometric technique and revealed by the appearance of a zone of discoloration in the dark background. *S. aureus* strains (spots 1 to 14): 1, ATCC 29213; 2, ATCC 25923; 3, a90; 4, a53; 5, a28; 6, a3; 7, a56; 8, a577; 9, a404; 10, a217; 11, a82; 12, a15; 13, a263; and 14, a40. A crude extract of *E. coli* C600(R46), which produces OXA-2 β-lactamase, was used as a positive control (spot 15).

DISCUSSION

Of the 10 epidemiologically independent *S. aureus* test strains selected on the basis of their borderline PRP suscepti-

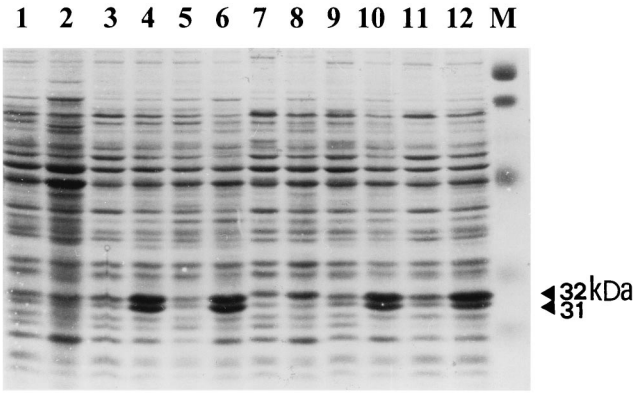

FIG. 2. Membrane protein patterns of six *S. aureus* strains (Coomassie blue-stained gel). Lanes: 1, 3, 5, 7, 9, and 11, strains a90, a3, a28, a40, a56, and a53, respectively, uninduced; 2, 4, 6, 8, 10, and 12, the same strains grown in the presence of methicillin (0.5 μg/ml); M, molecular mass markers (prestained SDS-PAGE standards, low range [Bio-Rad]).

bility, 9 behaved quite homogeneously and proved to be very similar both to one another and to a53, a previously investigated borderline *S. aureus* isolate (14, 15). Besides borderline PRP susceptibility, these strains shared a particular 17.2-kb β-lactamase plasmid, two distinct inducible β-lactamase bands in their membrane protein patterns, and membrane-associated hydrolytic activity against PRPs and PADAC. By contrast, the remaining borderline test strain (a40) did not share any of these particular characteristics and, unlike the other borderline test strains, was ultimately shown to produce PBP 2a. Although we were not able to determine the phage type of our test strains, investigations of strains belonging to the 94/96 complex confirmed reported relationships between this particular phage group and the borderline phenotype (19, 34), with the associated 17.2-kb β-lactamase plasmid (2, 14, 19), and suggested that this correlation also applies to methicillinase production. It is worth noting that the group V phage complex (which includes bacteriophages 94 and 96) is regarded as a quite distinct and homogeneous class of *S. aureus* strains, worldwide in occurrence, with a possible common origin (1, 22), and correlating with the production of large amounts of β-lactamase (23). Such a homogeneity may reflect the function of the restriction and modification systems in these strains that prevent the acquisition of genetic material from strains outside the complex (1, 22).

According to the original hypothesis by McDougal and Thornsberry (18), some *S. aureus* strains that produce large amounts of β-lactamase may show borderline susceptibility or resistance to PRPs because the staphylococcal β-lactamase, when hyperproduced, would succeed in partially hydrolyzing these penicillins. It was soon realized, however, that β-lactamase hyperproduction cannot be regarded as the only mechanism involved and that production of large amounts of β-lactamase may be neither sufficient nor necessary to determine the borderline phenotype (2, 15, 26, 32). The recent discovery of an inducible methicillinase—produced in addition to the classical penicillinase—in the membrane fraction of borderline *S. aureus* strains suggested that another, more specific PRP-hydrolyzing β-lactamase is more likely to account for the borderline phenotype than an increased amount of the conventional penicillinase (15). The slow hydrolysis of methicillin by this methicillinase, suggested by the overnight incubation necessary to detect the reaction in renatured gels (15), is consistent with the fact that its presence results in only reduced susceptibility rather than true resistance. On the other hand, the staphylococcal penicillinase, even if hyperproduced—as is the case with *E. coli* DH5α(pAH12), which expresses the product of the *blaZ* gene (i.e., penicillinase) derived from the 17.2-kb plasmid of *S. aureus* a53 (14)—appears not to be per se capable of hydrolyzing methicillin. This indirectly indicates that penicillinase hyperproduction is unlikely to be the true mechanism responsible for the borderline phenotype. That increased production of β-lactamase which is nevertheless often observed in borderline *S. aureus* strains might result from the combined effect of the second β-lactamase (methicillinase) in addition to the conventional one (penicillinase) on some common substrates such as nitrocefin and penicillin G.

The occurrence of such a methicillin-hydrolyzing β-lactamase in borderline *S. aureus* strains was first documented by means of SDS-PAGE of membrane proteins followed by gel renaturation (15). In the present study, the new β-lactamase activity associated with borderline *S. aureus* strains has been investigated by both gel renaturation and agarose plate assays. Methicillinase had been shown to be inducible and active against penicillin G and nitrocefin, in addition to methicillin (15); now, it has proved to be active, too, against isoxazolyl penicillins and nafcillin and against PADAC, again only after induction. While the ability of methicillinase to hydrolyze PRPs other than methicillin was expected, the hydrolysis of PADAC appears to deserve additional attention. In fact, this chromogenic cephalosporin is regarded as a possible substrate of the β-lactamases produced by some gram-negative bacteria but virtually insensitive to the β-lactamase activity of staphylococci (11).

In borderline *S. aureus* strains, production of methicillinase

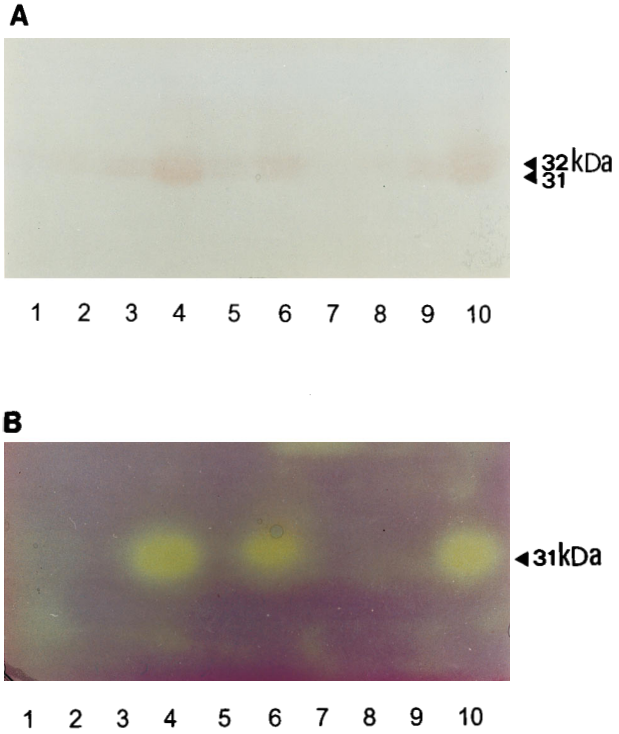

FIG. 3. Detection of β-lactamase activity in the membranes of five *S. aureus* strains by SDS-PAGE followed by gel renaturation. The chromogenic cephalosporins nitrocefin (A) and PADAC (B) were used as substrates. Lanes: 1, 3, 5, 7, and 9, strains a90, a3, a28, a40, and a56, respectively, uninduced; 2, 4, 6, 8, and 10, the same strains grown in the presence of methicillin (0.5 μg/ml).

is consistently associated with the presence of a 17.2-kb β-lactamase plasmid, containing the *blaZ* gene encoding penicillinase in the largest of the four *Hin*dIII fragments (14). An apparently identical β-lactamase plasmid, having the same size and the same *Hin*dIII restriction pattern, has been described by McMurray et al. (19) in borderline *S. aureus* strains. Barg et al. (2) reported transfer and curing of this plasmid from a borderline *S. aureus* strain belonging to phage group 94/96. In curing experiments, loss of the plasmid resulted in loss of borderline susceptibility. In transformation experiments, however, only the transformants obtained using a recipient of the 94/96 complex met the borderline susceptibility criteria, suggesting that the strain genetic background is important for the expression of the borderline phenotype. Considering its smaller size and other features, the 17.2-kb plasmid appears to be different from other β-lactamase plasmids of *S. aureus* (13, 25) and peculiar to borderline strains. Recently, we suggested that the 17.2-kb β-lactamase-encoding plasmid is the most likely candidate to encode methicillinase as well (14); in particular, we suggested that methicillinase could be the product of a mutated *blaR1* gene. In fact, the *blaR1* product (a signal-transducing membrane protein required for β-lactamase induction [6, 12]) has been shown to have high sequence similarity in *S. aureus* (24) as well as in *Bacillus licheniformis* (33) to the class D β-lactamases.

In the absence of official reference guidelines, determination of the limits of the borderline phenotype may be a problem (30). The relative lack of homogeneity in the criteria which have been adopted to define borderline *S. aureus* strains may cause difficulty in comparing the results of different microbiological and clinical studies involving these organisms. Of course, the reliability and the meaning itself of given limits for the borderline phenotype depend on the degree of correlation between the existence of particular levels of PRP MICs and the presence of particular biological characteristics. From this point of view, however, reduced staphylococcal susceptibility to PRPs may result from at least two categories of biological mechanisms, some extrinsic (depending on β-lactamase activity) and some intrinsic (depending on altered PBPs). The latter may be further associated with either the presence of PBP 2a (as reported previously [5, 28] and exemplified in this study by strain a40) or altered binding capacity of normal PBPs (as described in laboratory-derived [3, 29] and clinical [28] strains). According to the original description by McDougal and Thornsberry (18) and following studies (19, 20), including the present findings, extrinsic mechanisms are probably more common than intrinsic mechanisms among clinical isolates of borderline PRP-susceptible *S. aureus*. These extrinsic borderline strains appear to share, in addition to particular PRP MICs, a unique phage group, a unique β-lactamase plasmid, and a unique PRP-hydrolyzing β-lactamase (other than penicillinase and produced in addition to it as a membrane-associated enzyme). This methicillinase, in particular, is likely to be the direct cause of the reduced PRP susceptibility in these strains, so far regarded as β-lactamase hyperproducers. All this may give the concept of borderline PRP susceptibility a more precise and concrete biological meaning, rather than reducing it to a mere occurrence of given (more or less strict and predictive) MICs.

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