Identification of β-lactamases in human and bovine isolates of Staphylococcus aureus strains having borderline resistance to penicillinase-resistant penicillins (PRPs) with proteomic methods

Corresponding author: Ms. Judit Szilvia Keseru

E-mail address: kevia@freemail.hu

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aDepartment of Human Genetics, University of Debrecen, Debrecen, Hungary
bDepartment of Pharmacology and Pharmacotherapy, University of Debrecen, Debrecen, Hungary
cDepartment of Biomedical Science and Technology, Section of Medical Microbiology, University of Cagliari, Cagliari, Italy
dInstitute of Microbiology and Biomedical Sciences, Polytechnic University of Marche Medical School, Ancona, Italy
eCentral Veterinary Institute, Budapest, Hungary
fProteomics Research Group, Biological Research Center of the Hungarian Academy of Sciences, Szeged, Hungary
gDepartment of Pharmaceutical Chemistry, University of California, San Francisco, USA

Current addresses:
1Astrid Research Inc., Debrecen, Hungary
2Department of Biotechnology, Gedeon Richter Ltd., Budapest, Hungary
3Microbiological Research Group, National Center for Epidemiology, Budapest, Hungary

**Short title:** β-lactamases in *Staphylococcus aureus*
Corresponding author:

Judit Szilvia Keserü,

Department of Human Genetics,

Medical and Health Science Center,

Faculty of Medicine,

University of Debrecen,

Nagyerdei krt. 98.

Debrecen, Hungary, H-4032

Phone/Fax: 36-52-416-531

E-mail: kemia@freemail.hu
Abstract

Methicillin and oxacillin hydrolyzing enzymes of 6 borderline methicillin-resistant and 1 methicillin-resistant *Staphylococcus aureus* strains isolated from human clinical samples and 4 borderline methicillin-resistant *S. aureus* strains isolated from bovine mastitis were investigated. As previous studies suggested the involvement of an additional enzyme besides the penicillinase BlaZ in the determination of borderline resistance, we analyzed the expressed extracellular and membrane-bound β-lactamases with 2-D gel electrophoresis and mass spectrometry. Our analysis showed that the penicillin-hydrolyzing BlaZ alone was responsible for the hydrolysis of both methicillin and oxacillin. All supernatant and membrane fractions contained the same enzyme with slight sequence variations. The size and pI of the proteins were also variable, probably due to spontaneous hydrolysis and/or post-translational modifications. Interestingly, we found also cytotoxins and other virulence factors in some nitrocefin hydrolyzing dots, suggesting that those proteins might have a role in the reduction of local antibiotic concentration.

Key words: *Staphylococcus aureus*, borderline methicillin-resistance, membrane-bound β-lactamase, penicillinase, PRPs-hydrolysing enzyme, proteomics.
1. Introduction

Methicillin and the other penicillinase-resistant penicillins (PRPs), are narrow spectrum semisynthetic penicillins that share the property of being stable to the staphylococcal β-lactamase. Shortly after methicillin was introduced into clinical practice, methicillin-resistant *Staphylococcus aureus* (MRSA) strains emerged and spread rapidly since then (de Lencastre et al., 2007). In contrast to penicillin-resistance that is due to the production of β-lactamase, a penicillinase encoded by the *blaZ* gene, methicillin-resistance is due to the production of a penicillin-binding protein with reduced affinity to β-lactams, encoded by the *mecA* gene.

A distinct mechanism seems to account for the low-level or borderline resistance of *S. aureus* to methicillin and the other PRPs. Typical borderline methicillin-resistant *S. aureus* strains have an oxacillin MIC between 2 and 4 μg/ml, produce large amounts of β-lactamase and become susceptible to PRPs in the presence of β-lactamase inhibitors (Massidda et al., 1996). In addition, different from truly MRSA, that may show similar MICs to oxacillin, they do not carry the *mecA* gene. These β-lactamase hyperproducer strains harbour plasmids that encode a type A staphylococcal penicillinase, BlaZ (Chambers, 1997). The borderline phenotype was initially attributed solely to the hyperproduction of the BlaZ (McDougal and Thornsberry, 1986). Subsequent studies revealed that some borderline strains produce a second β-lactamase, a membrane-bound methicillinase, able to hydrolyse PRPs (Gáll et al., 2001; Kaszanyitzky et al., 2004; Keserü et al., 2005; Massidda et al., 1992). This enzymatic activity did not appear to be due to BlaZ (Massidda et al., 2006; Massidda et al., 1994, 1996).

In this paper our aim was to identify the PRP-hydrolyzing enzyme by proteomic analysis of the extracellular and membrane-bound β-lactamases of previously characterized borderline *S. aureus* strains isolated from clinical samples derived from different species.
2. Materials and Methods

2.1. Bacterial strains and growth conditions

10 borderline methicillin-resistant and 1 MRSA isolates (Table 1) were included in the study. The bovine isolates were selected from a previously described collection (Kaszanyitzky et al., 2004), accordingly to the guidelines specified by the CLSI (Clinical and Laboratory Standards Institute, former National Committee for Clinical Laboratory Standards, [NCCLS]) (National Committee for Clinical Laboratory Standards, 2000). The human isolates were selected according to the same criteria as bovine isolates as described (Gál et al., 2001; Kaszanyitzky et al., 2004; Keserű et al., 2005; Massidda et al., 1992). The discrimination between borderline methicillin-resistant strains that were truly MRSA was done testing \textit{mecA} (Table 1) as previously described (Gál et al., 2001; Keserű et al., 2005; Massidda et al., 1996).

Bacteria were grown in a 1% CY (casein – yeast) broth prepared as described (Novick and Brodsky, 1972) and supplemented with 0.5 $\mu$g/ml methicillin to induce $\beta$-lactamase production.

2.2. Preparation of bacterial supernatants

Cells were harvested by centrifugation at 4000 g for 30 min at 4$^\circ$C. 50 ml aliquots of supernatants were filtered via a Millipore sterile filter (0.45 $\mu$m) then concentrated with ultrafiltration (PM-10 membrane, Amicon) and with Centricon YM-3 Centrifugal Filter Units (Millipore).
2.3. Isolation of bacterial membranes

Harvested cells were washed twice with a 50 mM sodium-phosphate-buffer (pH 7.0), resuspended in the same buffer containing 1 mg/ml lysozyme (Sigma), then were incubated at 37°C for 1.5 h. The intact cells were pelleted by centrifugation at 1100 g for 10 min at 4°C and the protoplasts were collected by centrifuging the supernatant at 27000 g for 20 min at 4°C. The protoplasts were resuspended in distilled water for 5 min then 0.5 mg/ml MgCl₂ and 0.09 mg/ml DNase were added. The burst protoplasts were incubated at 37°C for 1 h then centrifuged at 3000 g for 10 min at 4°C. Cellular membrane fractions were collected by centrifugation of the supernatant at 27000 g for 20 min at 4°C, then washed three times with distilled water and stored at -20°C.

When the yield of that protocol was not satisfactory cells were sonicated by Branson Sonifier 250 (micro tip limit output control = 3; 50% duty cycle; on ice) then centrifuged at 3000 g for 10 min at 4°C to remove heavy cell degradation products. Cellular membrane fractions were collected also by centrifugation of the supernatant at 27000 g for 20 min at 4°C.

Because of the high lysozyme-resistance of strain a53 French press was used to disrupt the cells and whole cell lysate was used instead of membrane fraction.

2.4. 2-D gel electrophoresis of extracellular proteins

100-100 µg of concentrated proteins were precipitated and desalted using the ReadyPrep™2-D Cleanup Kit (Bio-Rad Laboratories), then redissolved in 125-125 µl of rehydration buffer (IEF buffer, sample buffer) containing 8 M urea, 2% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), 0.2% 3/10 IPG (immobilized pH
gradient) buffer (Bio-Rad Laboratories), 50 mM DTT (dithiothreitol) and 0.002% Bromophenol Blue. For first-dimension isoelectric focusing (IEF) 7 cm pH 3-10 IPG strips (Bio-Rad Laboratories) were rehydrated overnight in IEF buffer containing the sample at room temperature in a rehydration/equilibration tray. IEF was performed using Bio-Rad Protean IEF Cell. Proteins on the IPG strips were focused at 20°C at 4000 V to a total of 10000 Vh initiated by a slow voltage gradient from 0 to 250 V in 20 min, then from 250 V to 4000 V in 2 h. Following the IEF, IPG strips were processed for the second-dimension separation by a 10 min equilibration in 6 M urea, 2% SDS (sodium dodecyl sulfate, or sodium lauryl sulfate), 50 mM Tris-HCl (pH 8.8), 30% glycerol, 1% DTT followed by a second 10 min bath in a similar solution in which DTT was replaced by 2.5% iodoacetamide. Second dimension separation was performed essentially as described (Laemmli, 1970). Strips were applied to a Mini-Protean 2-D Cell (Bio-Rad Laboratories, Paris) with a 5% stacking gel, 13% separating gel and electrophoresis was performed at constant voltage of 200 V.

2.5. 2-D gel electrophoresis of membrane proteins

100-100 μg sample was desalted with ReadyPrep™ 2-D Cleanup Kit (Bio-Rad Laboratories, Paris) and was dissolved in maximum 50 μl rehydration buffer containing 7 M urea, 2 M thiourea, 2% CHAPS, 0.2% 3/10 IPG buffer, 50 mM DTT and 0.002% Bromophenol Blue.

Isoelectric focusing was performed using Bio-Rad Protean IEF Cell. 7 cm long pH 3-10 IPG strips were incubated for 16 h at room temperature with 135-135 μl sample buffer without sample. Samples were applied before IEF via cups at the anodic site. IPG strips were focused at 20 °C and the following voltage gradient was applied: from 0 to 250 V in 30 min, from 250 to 4000 V in 2 h and 4000 V to 10000 Vh.
In case of the whole cell lysate of strain a53 350 – 350 µg protein was applied to 17 cm long pH 3-10 strips in 100 µl. The strips were rehydrated in 330 – 330 µl of sample buffer. Samples were applied before IEF via cups at the anodic site. IPG strips were focused at 20 ºC and the following voltage gradient was applied: from 0 to 250 V in 30 min, from 250 to 10000 V in 2 h and 10000 V to 40000 Vh.

After isoelectric focusing the strips were handled as in the case of extracellular proteins.

2.6. Detection of β-lactamases on SDS-PA gels

Membrane and culture supernatant fractions (100 µg of total protein) were subjected to IEF and SDS-polyacrylamide gel electrophoresis (PAGE) in duplicates. One of the gels was soaked in a renaturating buffer for 6-8 h at 37 ºC (Massidda et al., 1992). Enzymatic activity was detected directly by placing: filter paper strips soaked in 1 mg/ml nitrocefin (O'Callaghan et al., 1972) solution on the appropriate areas of the gels corresponding to areas where we detected activities on 1-D SDS-PAGE previously (Kaszanyitzky et al., 2004; Keserű et al., 2005; Massidda et al., 1994). After the detection and marking of the β-lactamase dots or bands the gels were stained with Coomassie Brilliant Blue G-250 (Blue Silver) (Candiano et al., 2004).

The paralell gels were stained with Coomassie Brilliant Blue G-250 and appropriate spots were cut out a from the gels.

2.7. Mass spectrometric analysis

MALDI-TOF analysis was performed as described by Birkó et al. (Birkó et al., 2009). Peak picking was done manually, using X-Mass/X-Tof (ver.: 5.1.1) software from Bruker
Daltonics. Peak lists were subjected to database search either using ProteinProspector MS-Fit on-line search engine or Mascot.

LC-MS/MS analyses were carried out on an Agilent XCT Plus ion trap mass spectrometer on-line coupled with an Agilent 1100 nano-HPLC system. An aliquot of the samples were injected to a Zorbax 300SB-C18 5μm 5×0,3 mm trap column and washed onto the nano column (Zorbax 300SB-C18 3,5 μm 150 × 0,75 mm) using a short gradient elution of 5-45% solvent B in 20 min (flow rate: 300 nl/min, solvent A: 0.1% formic acid in water, solvent B: 0.1% formic acid in acetonitrile). Mass spectrometric analyses were carried out using information dependent mode acquiring the MS/MS spectra of the three most intense multiply charged precursor cations.

To identify the proteins present, mass spectrometry data were searched against the unrestricted NCBI protein database [NCBI nr 20070926 (5519594 sequences)] using the Mascot search engine. The parameters used for the database search identical to those described by Birkó et al. (Birkó et al., 2009). Protein lists were revised manually taking into consideration only the significant peptide hits (p<0.05), minimum two significant peptides/protein.

3. Results

3.1. Analysis of the extracellular β-lactamases

For the analysis of extracellular enzymes we used the conventional rehydration buffer and technique of sample preparation and of IEF. One pair of representative gel pictures (Fig. 1) shows the result obtained with the supernatant fraction of strain VU94. β-lactamase activity
appeared in wide areas of the gels (Fig. 1A). In most cases we found enzymes at pI 4, 5 and 9.

All active dots were analyzed (1-10 dots/gel), and the unambiguously identified proteins are
listed in Table 2. At least 42% of the significant peaks match with the database peptides of the
appropriate protein in case of MALDI-TOF, and at least 5 peptide fragments in case of LC-
MS/MS analysis.

In all cases BlaZ (gi|33416277) was identified as the extracellular β-lactamase (Table 2). In
strain 7789/47 a BlaZ variant (gi|3603441, erroneously called “type b” β-lactamase), and in
strain 7789/98 its longer variant (gi|67973147) was found together with BlaZ (gi|33416277)
or alone. The “type b” variants differed slightly (7%) from BlaZ because of the presence of a
peptide with molecular weight of 1440 Da corresponding to \textsuperscript{34}YNAN\textsubscript{IG}VYALDTK\textsuperscript{46} tryptic
fragment with an ion score of 89. In gi|33416277 BlaZ H is present instead of the underlined
N. In case of 7789/47 we also found a peptide with molecular weight of 1543 Da
corresponding to \textsuperscript{245}GQSEPI\textsubscript{LVIFTNK}\textsuperscript{258} (ion score was 87), which is present in
gi|33416277 BlaZ (in gi|3603441 it is not present, in gi|67973147 N is present instead of the
underlined G, and I is present instead of the underlined V). This probably represents a new
sequence variant of BlaZ in this strain. In strain 7789/98 a peak with molecular weight of
1973 (\textsuperscript{245}GQSEPI\textsubscript{LVIFTNKDNK}\textsuperscript{261}) instead of 1902 (\textsuperscript{245}NQSEPI\textsubscript{II}VIFTNKDNK\textsuperscript{261}) was
found, and also some other characteristic differences are labelled with asterisks on the
spectrograms on Supplemental Figure 1.

Besides BlaZ hemolysins (α-hemolysin in strains a53, a36, 9800, 9989, and 14287, β-
hemolysin in strains 822 and 14287, truncated β- hemolysin in strain 14287, γ-hemolysin in
strain 9989), some lipases (lipase 1 and 2 in strain 9800) and other cytotoxins and virulence
factors (leukocidin in strains a53 and a36, serine protease SplB in strains a53 and 14287/1)
were also able to hydrolyze nitrocefin (data not shown).
3.2. Analysis of the membrane-bound $\beta$-lactamases

In human samples the membrane-bound enzymes were attached to the membrane via a thioether-bond (Gál et al., 2001; Keserű et al., 2005), so we used a modified rehydration buffer and cup-loading technique to dissolve and apply the membrane proteins to the strips. One representative pair of gels (Fig. 2), shows the result obtained with the membrane fraction of strain VU94.

Similarly to the supernatant fractions we found enzymes at pI 4, 5 and 9 on the gels. All the active dots were analyzed (1-4 dots/gel), and the unambiguously identified proteins are listed in Table 3. At least 50% of the significant peaks match with the database peptides of the appropriate protein in case of MALDI-TOF, and at least 3 peptide fragments giving protein score larger than 25 in case of LC-MS/MS analysis.

All of the membrane fractions contained the same BlaZ (gi|33416277). In strains 7789/47 and 7789/98 a longer and a shorter “type b” BlaZ variant was found (gi|67973147 and gi|3603441, respectively). In case of strain 7789/98 we detected a peptide with molecular weight of 1097 Da, which corresponded to \textsuperscript{146}LGDKVTNPVR\textsuperscript{155} tryptic fragment with an ion score of 34. This can be produced only, when at position 145 a Lysine (gi|3603441, gi|67973147) is present and not Glutamic acid (gi|33416277). We also found a peptide with molecular weight of 1568 Da corresponding \textsuperscript{33}KYNA\textsubscript{N}GVIYALDTK\textsuperscript{46} (ion score was 82), which is present in gi|3603441 and in gi|67973147 BlaZ (in gi|33416277 H replaces the underlined N). This probably represents a new sequence variant of BlaZ in this strain. On the mass spectrogram of strain 7789/47 we found the same differences like in the case of the spectrogram of the extracellular enzyme of strain 7789/98, so probably both strains contain the same sequences variant.
4. Discussion

Our results show that all β-lactamase activities detectable in the supernatant and the membrane fractions of the borderline methicillin-resistant \textit{S. aureus} isolates, derived from human or bovine clinical samples, were all attributable to the BlaZ penicillinase produced by these strains. All BlaZ proteins detected are highly similar to each other, however slight sequence variations seem common and may account for the different size and, probably, the substrate specificity of the proteins (Voladri et al., 1996; Zawadzke et al., 1995). In strains 7789/47 and 7789/98 an additional enzyme with slight sequence variations was found.

Interestingly, in the supernatant fractions besides BlaZ that constitutes the main part of the β-lactamase activity detected, we found cytotoxins and other virulence factors in some nitrocefin hydrolyzing dots. It was established that some β-lactam antibiotics are able to trigger (Kernodle et al., 1995) and some β-lactamase inhibitors are able to inhibit the production of hemolysins (Tawfik et al., 1996). So, hemolysin production seems to be connected to the presence of β-lactams and thus may have a role in the reduction of local antibiotic concentration.

BlaZ was the only β-lactamase activity found in the membrane fractions and in the whole cell extract of the borderline methicillin-resistant \textit{S. aureus} strain a36 and a53, as shown in Fig. 2 (see spots M1 and M2, respectively). A likely explanation is that the two bands originally seen on SDS-PAGE renatured gels (Massidda et al., 1996; Massidda et al., 1992) contained the same protein with different sizes rather than two distinct β-lactamases. These results do not confirm the previous hypothesis proposing that a second β-lactamase, distinct from BlaZ, was responsible for the hydrolysis of PRPs in borderline strains (Massidda et al., 1996). However, the argument was based on the experimental findings that a recombinant \textit{E. coli} strain, constitutively expressing the \textit{blaZ} gene from a borderline strain, was not able to
hydrolyse PRPs (Massidda et al., 1996) and the blaZ gene showed a wild-type sequence (Massidda et al., 2006) and did not rule out different posttranslational modifications that could occur in a borderline methicillin-resistant background.

The different size and perhaps the substrate specificity of the same enzyme could be the consequence of the proteolysis of β-lactamases or posttranslational modification. A posttranslational modification (mainly phosphorylation) can explain the shift in the pI of the proteins. There are 23 Serine, 13 Threonine, and 13 Tyrosine residues in BlaZ, which can be phosphorylated. When 20 of them are phosphorylated it shifts the pI from 9.55 to 7 (result by ProMoST: Protein Modification Screening Tool, University of Wisconsin, Madison, Medical College of Wisconsin, Proteomics Center), so phosphorylation of all possible sites can shift the pI to 5 or 4 easily, and phosphate groups (MW: 95) also can cause an increase in the molecular weight of the protein. The 23 Asparagines and 4 Glutamines can be spontaneously hydrolyzed to Aspartic acid and Glutamic acid, respectively, which also can alter the pI (Zomber et al., 2005).

Bovine strains contained the same BlaZ β-lactamase like the human ones. Moreover, in two strains (7789/98 and 7789/47) we found an enzyme called erroneously type-b β-lactamase and its longer variant, having 7% sequence divergence compared to the gi|33416277 sequence. During the selection of the bovine strains also the phage type of them was established in the phage laboratory of the Hungarian National Public Health and Medical Officer Service, Debrecen, Hungary (unpublished data), and it showed that the 17599/3A and 17599/3B isolates could not been typed with bovine series of phages but only with the human series, suggesting that those strains may have a human origin. It is interesting that “type-b” β-lactamase or its longer variant was found only in strains with bovine origin. In these strains the shorter and longer variants of “type b” BlaZ were found alternatively in the supernatant or membrane fractions. It is also possible that the longer variant is present in all cases, but we
could not detect the C-terminal fragments. Because in the supernatant of strain 7789/47 a peptide also was identified that can be found in BlaZ (gi|33416277) we suppose that two enzymes are present in this strain, or it is more likely that a sequence variant that is not found in NCBI database is present in both strains.

5. Conclusion

In summary our results show that all ß-lactamase activities detectable in the supernatant and the membrane fractions of our borderline methicillin-resistant *S. aureus* isolates, derived from human or bovine clinical samples, were similar and all attributable to the BlaZ staphylococcal penicillinase produced by these strains. In many isolates an extracellular and a membrane-bound form of ß-lactamase were present. The extracellular form might have role in the reduction of local antibiotic concentration, while the membrane-bound enzyme could act at a more concentrated way. The “type-b” sequence variants were found only in the cow isolates. In two cases, it was shown that the bovine isolates have a human origin.

Acknowledgement

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Conflicts of interest

The authors have declared no conflicts of interest

References


Massidda, O., Montanari, M.P., Mingoia, M., Varaldo, P.E., 1996. Borderline methicillin-susceptible *Staphylococcus aureus* strains have more in common than reduced


Table 1. Methicillin-resistant and borderline methicillin-resistant *Staphylococcus aureus* isolates of human and animal origin

<table>
<thead>
<tr>
<th>Designation</th>
<th>Source</th>
<th>meca</th>
<th>β-lactamase production</th>
<th>Origin</th>
</tr>
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<tr>
<td>VU94</td>
<td>human</td>
<td>-</td>
<td>type A, extracellular and membrane-bound</td>
<td>provided by Dr. D. S. Kernodle (Vanderbilt University, Nashville, Tennessee, USA)</td>
</tr>
<tr>
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<td>human</td>
<td>-</td>
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<td>clinical isolate from the collection of the Institute of Microbiology, University of Ancona Medical School, Italy</td>
</tr>
<tr>
<td>822 (MRSA)</td>
<td>human</td>
<td>+</td>
<td>type A, extracellular and membrane-bound</td>
<td>clinical isolate from the collection of the Department of Microbiology, University of Debrecen, Hungary</td>
</tr>
<tr>
<td>9800</td>
<td>human</td>
<td>-</td>
<td>type A, extracellular and membrane-bound</td>
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</tr>
<tr>
<td>9989</td>
<td>human</td>
<td>-</td>
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</tr>
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<tr>
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<td>7789/98</td>
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Table 2. Nitrocefin-hydrolyzing extracellular enzymes of methicillin-resistant and borderline methicillin-resistant *Staphylococcus aureus* strains

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<tr>
<th>Sample(^1)</th>
<th>NCBI-GI</th>
<th>Protein name</th>
<th>Match(^2)</th>
<th>Seq. Cov. (%)</th>
<th>Nominal mass (M_r)</th>
<th>pI on gel</th>
<th>Theoretical pI</th>
<th>PSD or Mascot Score or MS/MS Search Score</th>
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<td>Beta-lactamase (BlaZ)</td>
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</table>

\(^1\)E1, E2, etc. correspond to the different active dots of the same gel

\(^2\)MALDI-TOF: Matched/observed masses, LC-MS: Total number of distinct peptides
3 Sequence covered
4 Mascot score
Table 3. Nitrocefin-hydrolyzing membrane-bound enzymes of methicillin-resistant and borderline methicillin-resistant *Staphylococcus aureus*

<table>
<thead>
<tr>
<th>Sample</th>
<th>NCBI-GI</th>
<th>Protein name</th>
<th>Match</th>
<th>Seq. Cov. %</th>
<th>Nominal mass Mₚ</th>
<th>pI on gel</th>
<th>Theoretical pI</th>
<th>PSD or MS/MS Search Score or Mascot Score</th>
</tr>
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</table>

1M1, M2, etc. correspond to different active dots of the same gel.
2 MALDI-TOF: Matched/observed masses, LC-MS: Total number of distinct peptides

3 Sequence covered

4 Different proteins having the same pI in the case of the same strains mean different observed molecular weight on the gel

5 Mascot score
Fig. 1. 2-D gel electrophoresis pattern of the supernatant fraction proteins of borderline methicillin-resistant *Staphylococcus aureus* strain VU94. (A): The gel was regenerated, and the nitrocefin hydrolyzing activities detected. (B): Blue Silver stained 2-D gel. The encircled areas are equivalent to the nitrocefin-hydrolyzing areas on (A). The + sign shows the acidic (pH 3) end.
Fig. 2. 2-D gel electrophoresis pattern of the membrane fraction proteins of borderline methicillin-resistant *Staphylococcus aureus* strain VU94. (A): The gel was regenerated, and the nitrocefin hydrolyzing activities detected. (B): Blue Silver stained 2-D gel. The encircled areas are equivalent to the nitrocefin-hydrolyzing areas on (A). The + sign shows the acidic (pH 3) end.
Suppl. Fig. 1.: Comparison of the spectra of BlaZ sequence variants. (A): Typical MALDI-TOF MS mass spectrum of gi|33416277 β-lactamase present in 14287/E3 sample. 38 matched out of 43 detected masses. The corresponding peptides covered 79 % of the protein sequence. (B): MALDI-TOF MS spectrum of 7789/98/E1 sample. 13 out of 16 detected peaks matched to the gi|67973147 beta-lactamase protein sequence. Sequence coverage was 44%. Asterisks show the peptides characteristic to the BlaZ sequence variants.