INTRODUCTION

The arginine catabolic mobile element (ACME) was first identified in the genomic sequence of meticillin-resistant Staphylococcus aureus (MRSA) USA300 in 2006 (Diep et al., 2006). ACME I is a striking feature of USA300, and plays an important role in its growth and survival, resulting in extensive dissemination (Diep et al., 2006; Diep & Otto, 2008).

ACME I is characterized by two gene clusters, arc and opp-3. The arc cluster, encoding a complete arginine deiminase pathway that converts L-arginine to carbon dioxide, ATP and ammonia, is of importance for survival at low pH and for the inhibition of the immune response against bacterial infections (Degnan et al., 2000; Moncada & Higgs, 1993). All S. aureus isolates have a native arc cluster encoding the arginine deiminase pathway, which differs from the ACME-arc cluster gene sequence and arrangement. Diep et al. (2006) presumed that the ACME-arc cluster contributed to the survival of USA300 by strengthening the function of arginine deiminase pathway. The presence of the opp-3 cluster, encoding a member of the ABC transporter family, is correlated with peptide nutrient uptake, quorum sensing, pheromone transport, chemotaxis, eukaryotic cell adhesion, binding of serum components and expression of virulence determinants (Podbielski et al., 1996). It was verified that deletion of ACME significantly attenuated the pathogenicity or fitness of USA300 (Diep et al., 2008).

ACME is integrated in orfX. In meticillin-resistant staphylococci, ACME is always adjacent to SCCmec elements, and its integration and excision might be catalysed by the SCCmec recombinase encoded by the ccr gene (Diep et al., 2006). To date, ACME-arcA genes have been identified in S. aureus (ST8-MSSA, ST8-MRSA-SCCmecIV, ST5-MRSA-SCCmecII, ST59-MRSA-SCCmecII, ST97-MRSA-SCCmecIV and ST1-MRSA-SCCmecV), Staphylococcus epidermidis and Staphylococcus capitis (Diep et al., 2006, 2008; Ellington et al., 2008; Goering et al., 2007).

Distribution of the ACME-arcA gene among meticillin-resistant Staphylococcus haemolyticus and identification of a novel ccr allotype in ACME-arcA-positive isolates

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The aim of this study was to investigate the prevalence and characteristics of ACME (arginine catabolic mobile element)-arcA-positive isolates among meticillin-resistant Staphylococcus haemolyticus (MRSH). ACME-arcA, native arcA and SCCmec elements were detected by PCR. Susceptibilities to 10 antimicrobial agents were compared between ACME-arcA-positive and -negative isolates by chi-square test. PFGE was used to investigate the clonal relatedness of ACME-arcA-positive isolates. The phylogenetic relationships of ACME-arcA and native arcA were analysed using the neighbour-joining methods of MEGA software. A total of 42 (47.7%) of 88 isolates distributed in 13 PFGE types were positive for the ACME-arcA gene. There were no significant differences in antimicrobial susceptibility between ACME-arcA-positive and -negative isolates. A novel ccr allotype (ccrAB<sub>3</sub>) was identified in ACME-arcA-positive isolates. Among 42 ACME-arcA-positive isolates: 8 isolates harboured SCCmec V, 8 isolates harbouring class C1 mec complex and ccr<sub>AB<sub>3</sub></sub>; 22 isolates harbouring class C1 mec complex and 4 isolates harbouring class C2 mec complex were negative for all known ccr allotypes. The ACME-arcA-positive isolates were first found in MRSH with high prevalence and clonal diversity, which suggests a mobility of ACME within MRSH. The results from this study revealed that MRSH is likely to be one of the potential reservoirs of ACME for Staphylococcus aureus.
The origin of ACME was still unknown. It has been assumed that ACME was excised from coagulase-negative staphylococci (CNS) by the ccr-encoded recombinase and then transferred horizontally to other staphylococci (Diep et al., 2006). Hence, we investigated the distribution of the ACME-arcA gene among meticillin-resistant Staphylococcus haemolyticus (MRSH) to learn more about ACME element in CNS. The ACME-arcA-positive isolates were further characterized by antibiogram, PFGE, SCCmec. Native arcA in MRS was also detected to explore its relationship with ACME-arcA.

METHODS

Bacterial isolates. Eighty-eight MRS isolates were collected from inpatients of the First Affiliated Hospital, College of Medicine, Zhejiang University, from April 2002 to April 2003. All isolates were identified to the species level using the API Staph test (bioMérieux). According to Clinical and Laboratory Standards Institute guidelines, the resistance to meticillin was determined by disc diffusion test of cefoxitin (Oxoid) and PCR detection of the mecA gene with primers as described by Kondo et al. (2007).

Susceptibility test. Penicillin, oxacillin, erythromycin, chloramphenicol, tetracycline, levofloxacin, vancomycin, teicoplanin, gentamicin and rifampicin (Oxoid) susceptibility were determined by disc diffusion method on Mueller–Hinton agar (bioMérieux) according to Clinical and Laboratory Standards Institute guidelines. S. aureus ATCC25923 was used as the quality control strain. Susceptibilities were compared between ACME-arcA-positive and -negative isolates by chi-square test.

PCR amplification and sequencing. Template DNA was prepared with a QIAamp DNA mini kit (Qiagen). Amplification of the ACME-arcA gene (624 bp) was performed with primers as described by Diep et al. (2006). Primers n_arcA.1 (5’-CGTCCAGCAGAAC-3’) and n_arcA.2 (5’-GACCCCAATCGCCTAAC-3’), designed on the basis of the sequence of S. haemolyticus strain JCSI1435 (GenBank accession number AP006716), were used to amplify native arcA (736 bp). The presence of SCCmec was determined by PCR amplifications of the mec complex (class A, B and C) and the ccr complex (ccrAB1, ccrAB2, ccrAB3, ccrAB4 and ccrC) with primers described by Katayama et al. (2001) and Kondo et al. (2007). Primers ccrA5 (5’-CAATGAC-TCACCGCAGCAG-3’) and ccrB5 (5’-CGTCTAGTTGGCCCATG-3’), designed in accordance with the sequence of Staphylococcus pseudintemedius strain KM241 (GenBank accession number AM904731) (Descloux et al., 2008), were used to amplify ccrAB5. Conserved primers for ccr allotypes 1–5, ccrA_up (5’-TGGATT-ATGTTGAGGAC-3’)/ccrA_dw (5’-CAATGTCGAGATGTGTTTTG-3’) and ccrB_up (5’-CTAGTTCAATCTGATTGGTC-3’)/ccrB_dw (5’-CATAGGTATGAGTGGTATG-3’) were used to amplify novel ccr allotypes in ccr-untypable isolates. A novel ccr allotype (ccrABISP) was found using these conserved primers and its complete sequence was further determined using a TaKaRa LA PCR in vitro cloning kit (Takara Bio). Primers ccrABISP.1 (5’-CGGCTCATTACGAGATCC-3’) and ccrBISP.1 (5’-CGGCTGATTACGAGATCC-3’) were used to detect the distribution of ccrABISP. All of the ampiclons (ACME-arcA, native arcA, mec complex and ccr complex) were sequenced using an ABI 3730 sequencer analyser (Applied Biosystems).

Dot blotting. Dot blotting using a DIG DNA labelling and detection kit (Roche Applied Science) was performed with PCR-generated probes specific for ccrAB1, ccrAB2, ccrAB3, ccrABISP and ccrC, according to the manufacturer’s instructions.

PFGE. Genomic DNA was prepared from the various isolates and digested with SmaI (Sangon) as described by Hanssen et al. (2004). DNA fragments were separated by electrophoresis in a 1 % agarose gel (Sangon) with a CHEF Mapper XA system (Bio-Rad) at 14 °C and 6 V cm⁻¹, and with alternating pulses at a 120° angle in a 3–40 s pulse time gradient for 22 h. The PFGE profiles were analysed using BioNumerics software (version 4.6) and interpreted by the criteria of Tenover et al. (1995).

Phylogenetic tree of arcA, ccrA and ccrB. ACME-arcA and native arcA (487 bp nucleotide fragments) identified in this study were compared with the following publicly available staphylococcal genomic sequences: S. aureus strains USA300_TCH1516 (GenBank accession number CP000730), Mu3 (AP009324), Newman (AP009351), JH1 (CP000736), JH9 (CP000703), Mu50 (BA000017), COL (CP000046), NCTC 8325 (CP000253), USA300_FPR3757 (CP000255), MSSA476 (BX571857), MW2 (BA000033), N315 (BA000018), RFI22 (AJ938182), and MRSA252 (BX571856); S. epidermidis strains ATCC12228 (AM015929) and RP62A (CP000029). S. haemolyticus strain JCSI1435 (AP006716); and Staphylococcus saprophyticus strain ATCC15305 (AP008934). The novel ccr allotype ccrABISP was compared with six known ccr allotypes: ccrAB1 (AB033763), ccrAB2 (D86934), ccrAB3 (AB037671), ccrAB4 (AF411935), ccrC (AB121219), ccrAB5 (AM904731), ccrABISP (AB353724) and ccrABISP (AP008934).

Phylogenetic trees of arcA, ccrA and ccrB were generated using the neighbour-joining methods of MEGA software (version 4.0). The topology of the phylogenetic trees was evaluated by bootstrap analyses with 1000 replicates to give the degree of confidence intervals for each node as indicated on the phylogenetic trees.

GenBank accession numbers. The complete sequence and annotation of the novel ccr allotype (ccrABISP) in S. haemolyticus have been deposited in the GenBank database (accession number EU934095).

RESULTS AND DISCUSSION

Distribution of the ACME-arcA gene in MRS

Besides S. aureus, ACME-arcA genes have also been identified in S. epidermidis and S. capitis. The high prevalence in S. epidermidis (18/27) suggested that ACME might be transferred into S. aureus from S. epidermidis and other CNS (Diep et al., 2006). However, Diep et al. (2006) did not find the ACME-arcA gene in seven S. haemolyticus isolates tested. That might be because of the limited number of isolates. In the present study, 42 (47.7 %) of 88 MRS isolates were positive for the ACME-arcA gene. This is believed to be the first report that the ACME-arcA gene is found in MRS. We compared the origin of specimens between ACME-arcA-positive and -negative isolates, to find whether the presence of ACME-arcA correlated with infection or colonization site. The ACME-arcA-positive isolates were obtained from different specimens, of which the majority (22/42) were from sputum, 6 were from blood, and 3 were from skin and soft tissue. The remaining 11 isolates were from throat swab (2), urine (2), venous catheter (2), peritoneal dialysis solution (2), urethral secretion (1), bile (1) and abdominal drain (1). With regard to ACME-arcA-negative isolates, 33 of 46 were
collected from sputum and 5 were from blood. The remaining eight isolates were from nasal swab, throat swab, bile, peritoneal dialysis solutions, abscess, urine, cerebrospinal fluid and surgery incision. No evidence was observed correlating the presence of ACME-arcA to the type of infection or colonization site.

Antimicrobial susceptibility

High resistance rates (>88%) of ACME-arcA-positive isolates to penicillin, oxacillin, erythromycin, levofloxacin and gentamicin were observed in this study. Tetracycline, chloramphenicol and rifampicin showed good activity against ACME-arcA-positive isolates, to which the resistance rates were lower than 25%. No ACME-arcA-positive isolates exhibited resistance to vancomycin and teicoplanin. Diep et al. (2006) proposed that the ACME element might be a new member of the SCC family, members of which are known to encode mobile genetic islands with the ability to carry various genes (Diep et al., 2006). To find whether antimicrobial resistance genes were carried by ACME elements in MRSH, antimicrobial susceptibility was compared between ACME-arcA-positive and -negative isolates by chi-square test. No significant difference was observed (Table 1). Likewise, no antimicrobial resistance gene was found in the complete sequence of ACME I in MRSA USA300 and ACME II in S. epidermidis ATCC12228 available from GenBank.

Genetic relationships

ACME-arcA positive isolates were distributed among 13 PFGE types (Fig. 1): pulsotype A (n=19), pulsotype B (n=2), pulsotype E (n=4), pulsotype G (n=2), pulsotype I (n=2), pulsotype J (n=3), pulsotype K (n=4). The other six isolates had different patterns to each other. A total of 19 of 42 ACME-arcA-positive isolates belonged to pulsotype A, making this the predominant pulsotype among these 42, as well as the predominant pulsotype among the 88 MRSH (data not shown). The high prevalence and genetic diversity of ACME-arcA-positive isolates was indicative of the mobility of ACME within this species.

Sequence analysis of ACME-arcA and native arcA

Forty-two amplicons (624 bp) of ACME-arcA were all sequenced and aligned using MEGA software. The nucleotide sequences of all the ACME-arcA amplicons were identical and showed high similarity (>99%) with their counterparts in USA300 and S. epidermidis ATCC12228. The native arcA amplicons (736 bp) obtained from 42 ACME-arcA-positive isolates were all sequenced too. Two native arcA allotypes with 99.6% nucleotide sequence identity were determined. The phylogenetic tree (Fig. 2) was constructed on the basis of the consensus sequence (487 bp) of ACME-arcA and native arcA, based on sequences obtained from this study and from the GenBank database. The arcA genes were clustered in two main groups, one containing ACME-arcA in three species of staphylococci and native arcA in S. haemolyticus, the other one containing native arcA in S. aureus and S. epidermidis. It was noteworthy that native arcA in S. haemolyticus had a closer relationship with ACME-arcA rather than with native arcA in S. aureus and S. epidermidis. These clues led to the proposal that ACME in S. aureus might be acquired horizontally from MRSH.

crr, SCCmec and ACME

The site-specific recombinase encoded by crr, a member of invertase/resolvase family, has been proved to be responsible for the integration and excision of SCCmec (Ito et al., 2001; Katayama et al., 2000). It is also assumed to be in charge of the movement of ACME (Diep et al., 2006). So far, five crr allotypes have been identified in MRSA, namely crrA1–4 and crrC (Descloux et al., 2008; Kondo et al., 2007). Multiple crr variants have also been found in CNS, such as crrAB5 in S. pseudintemedius, and crrAB15305 and crrAB53505 in S. saprophyticus (Descloux et al., 2008; Higashide et al., 2008; Kuroda et al., 2005). In the present study, a novel crr allotype was identified in ACME-arcA-positive isolates. The sizes of the two new recombinase-encoding genes designated crrASHP and crrBSHP were 1335 and 1647 bp, respectively. crrASHP showed 96% deduced amino acid sequence identity to crrAS in S. pseudintemedius and 78% to crrA3 in S. aureus. crrBSHP had 89 and 87% amino acid sequence identity to crrBS in S. pseudintemedius and crrB3 in S. aureus, respectively. We hypothesized that crrBSHP had a similar function to the known crr allotypes, and catalysed the mobility of SCCmec and ACME; this needs to be verified by excision and integration experiments. The phylogenetic trees of crrA and crrB are shown in Figs 3 and 4. There were eight ACME-

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<tr>
<th>Isolate</th>
<th>Penicillin</th>
<th>Oxacillin</th>
<th>Erythromycin</th>
<th>Levofloxacin</th>
<th>Gentamicin</th>
<th>Tetracycline</th>
<th>Chloramphenicol</th>
<th>Rifampicin</th>
<th>Vancomycin</th>
<th>Teicoplanin</th>
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<td>ACME-arcA-positive</td>
<td>100</td>
<td>100</td>
<td>95.2</td>
<td>92.9</td>
<td>88.1</td>
<td>23.8</td>
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<td>0</td>
<td>0</td>
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<tr>
<td>ACME-arcA-negative</td>
<td>100</td>
<td>100</td>
<td>95.6</td>
<td>95.6</td>
<td>84.4</td>
<td>8.9</td>
<td>33.3</td>
<td>11.1</td>
<td>0</td>
<td>0</td>
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<tr>
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<td>0.937</td>
<td>0.622</td>
<td>0.058</td>
<td>0.13</td>
<td>0.488</td>
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arcA-positive isolates harbouring ccrAB_{SHP}. Although these strains carried a class C1 mec gene complex, whether or not the mec gene complex was located in a chromosome cassette carrying novel ccr genes was not yet proved.

ACME has been associated with SCCmec II, IV and V in *S. aureus*, and with SCCpbp4 in *S. epidermidis* ATCC12228 (Diep *et al.*, 2006, 2008; Ellington *et al.*, 2008; Goering *et al.*, 2007). In this study, eight ACME-arcA-positive isolates harboured ccrAB_{SHP} and the class C1 mec complex, and eight isolates harboured SCCmec V. The remaining 22 isolates carried the class C1 mec complex and ccr-untypable (negative for all known ccr allotypes by PCR), and 4 isolates carried the class C2 mec complex and ccr-untypable.
In summary, ACME-arcA-positive isolates were found in MRSH, for what is believed to be the first time, with a high prevalence and clonal diversity, which is indicative of the mobility of ACME within this species and the transfer of ACME from MRSH to S. aureus. Furthermore, a novel ccr allele ccrAB<sub>SHP</sub> was identified in ACME-arcA-positive isolates, which might be responsible for the movement of SCCmec and ACME in MRSH.

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**REFERENCES**


