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

Borui Pi, Meihong Yu, Yagang Chen, Yunsong Yu and Lanjuan Li

Correspondence
Yunsong Yu
yyys119@163.com

State Key Laboratory for Diagnosis and Treatment of Infectious Disease, First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, Zhejiang 310003, PR China

The aim of this study was to investigate the prevalence and characteristics of ACME (arginine catabolic mobile element)-*arcA*-positive isolates among methicillin-resistant *Staphylococcus haemolyticus* (MRSH). ACME-*arcA*, native *arcA* and SCC*mec* 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*_{SHP}) was identified in ACME-*arcA*-positive isolates. Among 42 ACME-*arcA*-positive isolates: 8 isolates harboured SCC*mec* V, 8 isolates harboured class C1 *mec* complex and *ccrAB*_{SHP}; 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*.

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INTRODUCTION

The arginine catabolic mobile element (ACME) was first identified in the genomic sequence of methicillin-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 methicillin-resistant staphylococci, ACME is always adjacent to SCC*mec* elements, and its integration and excision might be catalysed by the SCC*mec* 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-SCC*mec*IV, ST5-MRSA-SCC*mec*II, ST59-MRSA-SCC*mec*II, ST97-MRSA-SCC*mec*V and ST1-MRSA-SCC*mec*IVa), *Staphylococcus epidermidis* and *Staphylococcus capitis* (Diep *et al.*, 2006, 2008; Ellington *et al.*, 2008; Goering *et al.*, 2007).

Abbreviations: ACME, arginine catabolic mobile element; CNS, coagulase-negative staphylococci; MRSA, methicillin-resistant *Staphylococcus aureus*; MRSH, methicillin-resistant *Staphylococcus haemolyticus*.

The GenBank/EMBL/DDBJ accession number for the novel *ccr* allotype (*ccrAB*_{SHP}) sequence of *Staphylococcus haemolyticus* is EU934095.

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 methicillin-resistant *Staphylococcus haemolyticus* (MRSH) to learn more about ACME element in CNS. The ACME-*arcA*-positive isolates were further characterized by antibiogram, PFGE, SCC*mec*. Native *arcA* in MRSH was also detected to explore its relationship with ACME-*arcA*.

METHODS

Bacterial isolates. Eighty-eight MRSH 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 methicillin 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'-CGTCCACGAACAAGT-3') and *n_arcA_2* (5'-GACCCAATGCCTAAC-3'), designed on the basis of the sequence of *S. haemolyticus* strain JCSC1435 (GenBank accession number AP006716), were used to amplify native *arcA* (736 bp). The presence of SCC*mec* 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'-CAATCAC-TACCGCACAG-3') and *ccrB5* (5'-CGTCTAGTTTGGCCCATG-3'), designed in accordance with the sequence of *Staphylococcus pseudintermedius* 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'-TGCATTC-ATGTTTGGAGAC-3')/*ccrA_dw* (5'-CAATGTGACGTATTGTGTTG-3') and *ccrB_up* (5'-GTTCTTTACCATGGACTTG-3')/*ccrB_dw* (5'-CTAGAAGGCTACTATCAAGG-3'), were used to amplify novel *ccr* allotypes in *ccr*-untypable isolates. A novel *ccr* allotype (*ccrAB_{SHP}*) 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 *ccrA_{SHP}* (5'-CGCGCTATTATCACGAATCC-3') and *ccrB_{SHP}* (5'-GCGTGATTAAGTGCCTAGC-3') were used to detect the distribution of *ccrAB_{SHP}*. All of the amplicons (ACME-*arcA*, native *arcA*, *mec* complex and *ccr* complex) were sequenced using an ABI 3730 sequence 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*, *ccrAB_{SHP}* and *ccrC*, according to the manufacturer's instructions.

PFGE. Genomic DNA was prepared from the various isolates and digested with *Sma*I (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), RF122 (AJ938182), and MRSA252 (BX571856); *S. epidermidis* strains ATCC12228 (AE015929) and RP62A (CP000029); *S. haemolyticus* strain JCSC1435 (AP006716); and *Staphylococcus saprophyticus* strain ATCC15305 (AP008934). The novel *ccr* allotype *ccrAB_{SHP}* was compared with six known *ccr* allotypes: *ccrAB1* (AB033763), *ccrAB2* (D86934), *ccrAB3* (AB037671), *ccrAB4* (AF411935), *ccrC* (AB121219), *ccrAB5* (AM904731), *ccrAB_{STU33}* (AB353724) and *ccrAB₁₅₃₀₅* (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 (*ccrAB_{SHP}*) in *S. haemolyticus* have been deposited in the GenBank database (accession number EU934095).

RESULTS AND DISCUSSION

Distribution of the ACME-*arcA* gene in MRSH

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 MRSH isolates were positive for the ACME-*arcA* gene. This is believed to be the first report that the ACME-*arcA* gene is found in MRSH. 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.

ccr, SCCmec and ACME

The site-specific recombinase encoded by *ccr*, 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 *ccr* allotypes have been identified in MRSA, namely *ccrAB1-4* and *ccrC* (Descoux *et al.*, 2008; Kondo *et al.*, 2007). Multiple *ccr* variants have also been found in CNS, such as *ccrAB5* in *S. pseudintermedius*, and *ccrAB*_{STU33} and *ccrAB*₁₅₃₀₅ in *S. saprophyticus* (Descoux *et al.*, 2008; Higashide *et al.*, 2008; Kuroda *et al.*, 2005). In the present study, a novel *ccr* allotype was identified in ACME-*arcA*-positive isolates. The sizes of the two new recombinase-encoding genes designated *ccrA*_{SHP} and *ccrB*_{SHP} were 1335 and 1647 bp, respectively. *ccrA*_{SHP} showed 86% deduced amino acid sequence identity to *ccrA5* in *S. pseudintermedius* and 78% to *ccrA3* in *S. aureus*. *ccrB*_{SHP} had 89 and 87% amino acid sequence identity to *ccrB5* in *S. pseudintermedius* and *ccrB3* in *S. aureus*, respectively. We hypothesized that *ccrAB*_{SHP} had a similar function to the known *ccr* allotypes, and catalysed the mobility of SCCmec and ACME; this needs to be verified by excision and integration experiments. The phylogenetic trees of *ccrA* and *ccrB* are shown in Figs 3 and 4. There were eight ACME-

Table 1. Antimicrobial susceptibility of ACME-*arcA*-positive and -negative isolates

Isolate	Resistance rate (%)									
	Penicillin	Oxacillin	Erythromycin	Levofloxacin	Gentamicin	Tetracycline	Chloramphenicol	Rifampicin	Vancomycin	Teicoplanin
ACME- <i>arcA</i> -positive	100	100	95.2	92.9	88.1	23.8	19	4.8	0	0
ACME- <i>arcA</i> -negative	100	100	95.6	95.6	84.4	8.9	33.3	11.1	0	0
<i>P</i> value			1	0.937	0.622	0.058	0.13	0.488

Fig. 1. PFGE profiles of ACME-*arcA*-positive isolates.

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 SCC*mec* II, IV and V in *S. aureus*, and with SCC*pbp4* 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 SCC*mec* 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. The

Fig. 2. Phylogenetic tree of *arcA* (487 bp nucleotide fragments).

Fig. 3. Phylogenetic tree of *ccrA*.

ccr-untypable isolates were confirmed by PCR with conserved primers specific for *ccr* allotypes 1–5 and by dot-blot hybridization with probes specific for *ccrAB*_{1–3}, *ccrAB*_{SHP} and *ccrC*. Dot-blot hybridization was not performed with *ccrAB*₄ and *ccrAB*₅, due to the lack of a positive control. Most isolates (26/42, 61.9%) were still negative for all known *ccr* allotypes. Thus we presumed that there might be other novel *ccr* allotypes responsible for the movement of SCC*mec* and ACME in MRSH.

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* allotype *ccrAB*_{SHP} was identified in ACME-*arcA*-positive isolates, which might be responsible for the movement of ACME.

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Fig. 4. Phylogenetic tree of *ccrB*.

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