

Arginine Catabolic Mobile Element Is Associated With Low Antibiotic Resistance and Low Pathogenicity in *Staphylococcus epidermidis* From Neonates

HILDEGUNN N. GRANSLO, CLAUS KLINGENBERG, ELIZABETH G. A. FREDHEIM, ARILD RØNNESTAD, TOM E. MOLLNES, AND TROND FLÆGSTAD

Department of Pediatrics [H.N.G., C.K., E.G.A.F., T.F.], University of Tromsø, N-9037 Tromsø, Norway; Department of Pediatrics [C.K., T.F.], University Hospital of North Norway, N-9038 Tromsø, Norway; Department of Pediatrics [A.R.], Oslo University Hospital, Rikshospitalet, N-0027 Oslo, Norway; Department of Immunology and Transfusion Medicine [T.E.M.], Nordland Hospital, N-8092 Bodø, Norway; Department of Medical Biology [T.E.M.], University of Tromsø, Tromsø, Norway

ABSTRACT: The arginine catabolic mobile element (ACME) in *Staphylococci* encodes several putative virulence factors. ACME appears to have been transferred from *Staphylococcus epidermidis* into *Staphylococcus aureus* and is strongly associated with the epidemic and virulent *S. aureus* USA300. We sought to determine the distribution of ACME in 128 *S. epidermidis* blood culture isolates from neonates and to assess ACME's impact on antibiotic resistance, biofilm production, invasive capacity, and host inflammatory response. ACME was detected in 15/64 (23%) invasive blood culture isolates and 26/64 (40%) blood culture contaminants ($p = 0.02$). ACME-positive *S. epidermidis* isolates displayed less antibiotic resistance ($p < 0.001$) and were collected from more mature neonates ($p = 0.001$). Biofilm production was more prevalent among ACME-negative isolates (61/87) compared with ACME positive (18/41; $p = 0.004$). Among the 64 children considered having an invasive infection, ACME did not influence the maximum C-reactive protein level. In an *in vitro* whole-blood sepsis model, there were no differences in the inflammatory response between ACME-positive and ACME-negative isolates. We conclude that ACME in *S. epidermidis* from neonates was associated with less antibiotic resistance and also does not seem to be associated with increased pathogenicity. (*Pediatr Res* 68: 237–241, 2010)

Staphylococcus epidermidis is a frequent cause of late onset sepsis in preterm neonates (1,2). The major virulence factor of *S. epidermidis* is biofilm formation on polymer surfaces, *i.e.* indwelling central venous lines (3). A wide range of other putative virulence factors, *e.g.* surface proteins (4), phenol soluble modulins (PSM), and poly- γ -glutamic acid (3,5–7), have also been identified. However, their clinical relevance is yet to be proven.

The arginine catabolic mobile element (ACME) is a genomic island in *Staphylococci* that may contribute to enhanced pathogenicity. It was first discovered in the community associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) USA300 strain and the biofilm-negative *S. epidermidis* strain ATCC12228 (8). ACME contains two characteristic

gene clusters (the *arc*-operon and the *opp3*-operon) that are homologs of virulence determinants in other bacterial species. The *arc*-operon, a characteristic cluster of six genes, encodes several enzymes in the arginine deiminase catabolic pathway, a putative virulence pathway that convert L-arginine to carbon dioxide, carbamoyl ornithine, ammonia, and ATP (9,10). The *opp3*-operon encodes an oligopeptide permease system. Similar *opp*-operons in other bacterial species have a wide array of functions including pheromone transport, chemotaxis, and expression of virulence determinants (8). ACME has been divided into three allotypes (8–11). ACME-I, first found in *S. aureus* USA300, possess the *arc*-operon and the *opp3*-operon. ACME-II, first found in *S. epidermidis* ATCC12228, consists mainly of the *arc*-operon. ACME-III has only the *opp3*-operon and lacks the *arc*-operon. All three allotypes have been detected in *S. epidermidis* (11).

ACME is associated with the geographically widespread *S. aureus* clone USA300 and is believed to be a survival factor for this species (8,12). One hypothesis is that a decrease in the host inflammatory response is mediated by arginine deiminase inhibition of mononuclear cells (13) and through a depletion of L-arginine, the substrate for nitric oxide production (8). Previous studies have shown a high prevalence of ACME among *S. epidermidis* isolates, indicating that ACME might confer an advantage also to this species (8,9,11).

To our knowledge, no studies have evaluated the inflammatory response to *S. epidermidis* with regard to the presence or absence of ACME. First, the aim of this study was to evaluate the presence of all three ACME allotypes in *S. epidermidis* blood culture isolates obtained from neonates and second, to assess antibiotic resistance and compare different components of the inflammatory response in ACME-positive and ACME-negative isolates.

Abbreviations: ACME, arginine catabolic mobile element; CoNS, coagulase-negative *Staphylococci*; CRP, C-reactive protein; MLST, multilocus sequence typing; MRSA, methicillin-resistant *Staphylococcus aureus*; PFGE, pulsed field gel electrophoresis; SCC, staphylococcal chromosome cassette

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Correspondence: Claus Klingenberg, M.D., Ph.D., Department of Pediatrics, University Hospital of North Norway, N-9038 Tromsø, Norway; e-mail: claus.klingenberg@unn.no
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MATERIALS AND METHODS

A collection of 128 *S. epidermidis* blood culture isolates obtained from patients admitted to the neonatal intensive care unit at Oslo University Hospital, Rikshospitalet, were investigated. Blood culture isolates that were accompanied by clinical signs of sepsis in a neonate older than 72 h of age, and with a C-reactive protein (CRP) level >10 mg/L were considered as true invasive isolates. All other isolates were considered contaminants. Clinical and microbiologic data, including molecular genetic typing [pulsed field gel electrophoresis (PFGE) and multilocus sequence typing (MLST)] have been published previously (14,15). PFGE patterns were analyzed by GelCompar II version 2.5 (Applied Maths, Belgium). Isolates with ≥95% similarity were considered to be indistinguishable strains and isolates with ≥80% similarity were considered to be related strains (14,15).

We determined the minimal inhibitory concentration (MIC) values of oxacillin, gentamicin, fusidic acid, clindamycin, and vancomycin with Etest (AB Biodisk, Solna, Sweden) and ciprofloxacin by disk diffusion test. Antibiotic susceptibility was interpreted according to the EUCAST clinical breakpoints (16). Semiquantitative determination of biofilm production in microtitre plates was performed and interpreted as previously described (14,17). *S. epidermidis* ATCC 35984 (RP62A) and *S. epidermidis* ATCC 12228 served as positive and negative controls, respectively. We performed PCRs using previously reported primers for (i) the methicillin resistance gene (*mecA*) (18), (ii) one central aminoglycoside resistance gene [*aac(6')*-*Ie-aph(2')*-*Ia*] (19), (iii) a marker of the *ica*-operon encoding biofilm accumulation (*icaD*) (20), and (iv) ACME-*arcA* and ACME-*opp3* (8). Purified PCR products of two ACME-*opp3* and two ACME-*arcA* positive strains, respectively, were sequenced (BigDye Terminator, version 3.1; Applied Biosystems, Warrington) and analyzed on an ABI Prism 377 sequence analyzer (Applied Biosystems, Foster City, CA). To assess a possible association between ACME and staphylococcal chromosome cassette (SCC) elements in *S. epidermidis*, all ACME-positive isolates were further analyzed with a multiplex PCR (eight loci A-H), as previously described (21).

We selected eight of the ACME-positive and eight of the ACME-negative isolates for further experiments using a whole-blood model to assess *in vitro* inflammatory response. All 16 isolates were genetically distinguishable by PFGE. We minimized differences between the groups in potentially confounding factors, notably variables, which were previously shown to influence inflammatory response (biofilm production and methicillin resistance) (14). We also had equal numbers of invasive and noninvasive strains in each group. Each experiment was run in two parallels with blood from one healthy volunteer. Blood was collected into sterile polypropylene tubes (4.5 mL Nunc cryotubes; Nalgene NUNC, Roskilde, Denmark) containing 50 µg/mL lepirudin (Refludan, Pharmion ApS, Copenhagen, Denmark). Lepirudin, a recombinant hirudin analog with highly specific thrombin inhibitory activity, was used as anticoagulant because it has minimal influence on complement activation (22). The blood samples were obtained immediately before each experiment and kept at 37°C. Bacteria were added to create a blood-bacteria solution containing ~10⁸ colony forming units (CFU)/mL, verified by spreading diluted inoculum on blood-agar plates and determining CFU count. Oxidative burst and CD11b expression, indicating leukocyte activation, was analyzed in a flow cytometer. Six hundred microliters of the blood-bacteria solution was used for both these analyses after an incubation period of 10 min at 37°C. Median fluorescence intensity (MFI) of oxidative-burst leukocytes was measured according to the manufacturer's instructions with the Phagoburst kit (ORPEGEN, Heidelberg, Germany) on a FACSAria flow cytometer (Becton Dickinson, San Jose, CA). CD11b expression was measured on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) as described previously (22).

A multiplex assay (Bioplex cytokine assay; Bio-Rad, Hercules, CA) was used to quantify proinflammatory cytokines and chemokines (TNF-α, IL-1β, MIP-1α, IL-6, and IL-8) according to the manufacturer's instructions. In short, 1.8 mL of the blood-bacteria solution was transferred to segments of polyvinyl chloride (PVC) tubing (length 30 cm, internal diameter 3 mm, Medioplast, Malmö, Sweden). Each segment was closed end-to-end and formed small loops. The loops were rotated slowly in an incubator at 37°C for 3 h (22,23). After 3 h, plasma was separated, and proinflammatory cytokines and chemokines were analyzed. Soluble terminal C5b-9 complement complex (TCC) was analyzed in plasma by an ELISA kit (HK328; Hycult Biotechnologies, Uden, Netherlands) using the same technique as for the cytokine assay but with a 30-min incubation. EDTA was added (final concentration of 10 mM) immediately after incubation.

The committee for human medical research ethics, Region North in Norway, approved the collection of bacterial isolates, analysis of patient data, and collection of blood for the immune response study. Informed written consent was obtained from each blood donor.

The Mann-Whitney test was used to compare the differences between continuous variables without normal distribution and the χ² test or Fisher's exact, as appropriate, were used for dichotomous variables. Among the neonates with *S. epidermidis* sepsis (invasive isolates), we performed a linear multivariable regression analysis to identify bacterial and clinical variables potentially influencing CRP, as a marker of inflammatory response. CRP values were log transformed to reduce skewness. Two-sample *t* test was used for the *in vitro* inflammatory response study. All analyses were two tailed, and *p* < 0.05 was considered to be significant. Statistical analysis was performed with SPSS for Windows software version 16.0 (Chicago, IL).

RESULTS

Sixty-four isolates were considered as true invasive isolates, and 64 isolates were considered contaminants based on our predefined criteria (Table 1). Forty-one of 128 (32%) isolates were ACME positive; 20 ACME-I, 17 ACME-II, and 4 ACME-III. Table 1 shows the distribution of ACME-positive and ACME-negative isolates among invasive and contaminant *S. epidermidis* blood culture isolates. Twenty-six of 64 (40%) blood culture contaminants were ACME positive, whereas only 15 of 64 (23%) invasive isolates were ACME positive (*p* = 0.02). ACME-positive blood culture contaminants were collected from a group of neonates that had a higher birth weight and GA compared with neonates with ACME-negative contaminants (Table 1). There was no difference in the inflammatory response (CRP) in patients with ACME-positive invasive isolates compared with patients with ACME-negative invasive isolates (Table 1). A post hoc linear multivariable regression analysis adjusting for possible confounders (biofilm production, methicillin resistance, GA, and age at onset infection) also failed to show any influence of ACME on neonatal inflammatory response (data not shown).

Table 1. *S. epidermidis* blood culture isolates; ACME and relation to clinical data

	Invasive (n = 64)		<i>p</i>	Contaminants (n = 64)		<i>p</i>
	ACME-positive (n = 15)	ACME-negative (n = 49)		ACME-positive (n = 26)	ACME-negative (n = 38)	
Clinical data						
GA (wk)	28 (27–40)	27 (26–32)	0.36	40 (31–40)	28 (26–35)	0.001
Birth weight (g)	1120 (750–3410)	960 (701–1783)	0.36	2810 (1351–3670)	970 (790–2230)	0.001
CRP (mg/L)	54 (18–114)	47 (34–82)	0.92	NP*	NP	

Data are presented as median (interquartile range).

* NP, Not presented (data not presented as CRP <10 mg/L is one of the criteria defining an isolate as a contaminant).

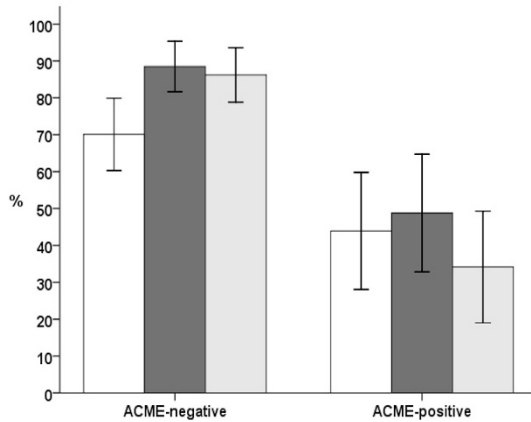


Figure 1. Biofilm production and methicillin- and gentamicin resistance among ACME-positive and ACME-negative blood culture isolates ($n = 128$) obtained from neonates. □, biofilm positive; ■, methicillin resistance; and ▤, gentamicin resistance. *T* bars represent 95% confidence intervals.

Molecular typing with PFGE showed two large clusters of related *S. epidermidis* isolates (15 strains PFGE-type 1 and 35 strains PFGE-type 27) comprising 50 of the 128 neonatal blood culture isolates. Molecular typing with MLST confirmed that these cluster isolates were related and that the vast majority of them belonged to a clonal complex, designated ST2 (15). Only two of 50 cluster isolates were ACME-positive. The other 39 ACME-positive isolates were evenly distributed among 78 sporadic, nonrelated *S. epidermidis* isolates. Sequencing ACME-*arcA* and ACME-*opp3* from four isolates verified that the gene products were the same as those previously published (8,11). SCCmec typing of the 41 ACME-positive isolates revealed 2 isolates with SCCmec type IV, 33 isolates with nontypeable SCC elements, and in 6 isolates no SCC elements were detected.

Among all 128 isolates, methicillin resistance ($p < 0.001$), gentamicin resistance ($p < 0.001$), and biofilm production ($p = 0.004$) were more prevalent among ACME-negative isolates compared with ACME-positive isolates (Fig. 1). None of the strains were resistant to vancomycin. To exclude impact of clustering, we did a subgroup analysis of the 78 sporadic nonrelated isolates. ACME-negative isolates still displayed significant higher methicillin resistance (29/39 versus 19/39, $p = 0.035$) and gentamicin resistance (27/39 versus 13/39, $p = 0.003$) than ACME-positive isolates, but there were no longer significant difference in biofilm production (data not shown). Among sporadic isolates, 20/39 ACME-negative and 14/39 ACME-positive isolates were considered invasive ($p = 0.17$).

Results from the whole-blood model of inflammatory response to *S. epidermidis* are shown in Table 2. We found no significant differences between ACME-positive and -negative strains for a broad range of inflammatory response pathways.

DISCUSSION

We found a high prevalence of ACME in *S. epidermidis* isolates from neonates. Our finding is in line with the study by Diep *et al.* (8), in which 67% (18/27) of *S. epidermidis* isolates from nostrils were ACME positive, and Miragaia *et al.* (11), in

Table 2. Inflammatory response measurements for eight ACME-positive and eight ACME-negative *S. epidermidis* isolates in a human whole-blood model

	ACME positive	ACME negative	<i>p</i>
Oxidative burst			
Granulocyte burst*	51 (14)	43 (18)	0.323
Granulocyte (MFI)	307 (111)	238 (140)	0.296
Monocyte burst*	28 (14)	21 (12)	0.303
Monocyte (MFI)	139 (64)	104 (38)	0.207
CD11b expression			
Granulocytes (MFI)	49,183 (12093)	53,553 (9016)	0.426
Monocyte (MFI)	12,976 (4931)	12,920 (5504)	0.983
TCC (Au/mL)	113 (23)	111 (21)	0.852
Cytokines			
TNF- α (pg/mL)	41,713 (11862)	47,627 (13690)	0.371
MIP-1 α (pg/mL)	908 (228)	1,104 (259)	0.131
IL-8 (pg/mL)	7,758 (2846)	9,174 (4765)	0.483
IL-6 (pg/mL)	2,994 (1296)	3,365 (1291)	0.575
IL-1 β (pg/mL)	3,294 (1709)	4,259 (2/996)	0.189

All 16 isolates are biofilm producers (*icaD*-positive) and oxacillin resistant (*mecA*-positive).

MFI, median fluorescence intensity; TCC, terminal complement complex.

Data presented as mean (SD).

* Percent cells burst/total cells.

which 51% (65/127) of *S. epidermidis* isolates from different sources and widespread geographical origin were ACME-positive. A recent study also showed a high prevalence (48%) of ACME in *Staphylococcus haemolyticus* isolates (24). In this study, a higher prevalence of ACME was found in *S. epidermidis* isolates considered as contaminants (most likely derived from the skin) compared with invasive disease causing isolates. It has been suggested that L-arginine catabolism contributes to optimize staphylococcal life on the acidic human skin, which may explain the high prevalence of ACME in typically skin colonizing coagulase-negative *Staphylococci* (CoNS), such as *S. epidermidis* and *S. haemolyticus* (10,24). Transfer of ACME from CoNS to the USA300 MRSA clone may also explain why USA300 frequently is colonizing skin regions (*e.g.* axilla and inguinal) not previously considered as typical niches for MRSA colonization (11,25).

In *S. aureus*, the role of ACME on virulence is unclear. In a rabbit MRSA bacteremia model, the ACME deletion mutant showed attenuated bacterial infectivity (9), whereas in a rat model of MRSA pneumonia, ACME had no impact on virulence (26). One epidemiologic study of *S. epidermidis* found no association between ACME and disease causing isolates, but clinical details were not presented (11). Our study did not identify any association between presence of ACME and invasive neonatal *S. epidermidis* infections. We noted that the mean GA of infants with contaminant isolates was higher than of infants with invasive blood culture isolates ($p = 0.02$), most likely reflecting that preterm infants have an impaired immune system and higher risk acquiring invasive *S. epidermidis* infections (27). ACME was frequently found in contaminant isolates from mature term-born babies, and we speculate that ACME in *S. epidermidis* may be considered as an indicator of benign, skin flora isolates.

To our knowledge, no previous data has been presented on the correlation between ACME and inflammatory response to

S. epidermidis. We have previously shown that biofilm-positive CoNS sepsis isolates induced a lower neonatal CRP response than biofilm-negative isolates. In contrast, methicillin resistance was associated with higher inflammatory response, most likely because of the delay in adequate antibiotic therapy (14). In our analysis of 64 episodes of *S. epidermidis* neonatal sepsis (invasive isolates), the presence or absence of ACME did not influence neonatal inflammatory response, even after adjusting for possible confounders. However, these results should be interpreted with caution as the limited number of ACME-positive isolates and high standard deviations of CRP levels only gave us a power to detect >50% difference in CRP levels. In the human whole-blood model, we also found no differences in a broad range of inflammatory responses between ACME-positive and ACME-negative isolates. This whole-blood model is developed specifically for studying the interaction of pathways of inflammation (22,28) and is considered a robust model for detecting differences in inflammatory response. Thus, in our study, ACME in *S. epidermidis* did not have any obvious impact on the host inflammatory response.

In *S. aureus*, ACME-positive isolates cluster in major MRSA-clonal lineages (9). Molecular typing of *S. epidermidis* blood culture isolates in our study revealed a different epidemiologic pattern, where ACME was associated with a high clonal diversity. In contrast, ACME was rarely present in the local endemic *S. epidermidis* clone affecting more immature infants in the neonatal intensive care unit (15). A similar high clonal diversity was also found among 88 ACME-positive *S. haemolyticus* isolates in a study from China (24). Miragaia *et al.*, in contrast, found that in geographically diverse *S. epidermidis* isolates, the vast majority of ACME containing isolates belonged to a large clonal complex. There is now compelling evidence that ACME is widely distributed among different CoNS-species, and that CoNS may act as a reservoir for ACME being transferred to specific clonal lineages of *S. aureus* (8,11,24).

Studies in *S. aureus* show that ACME is often integrated in the bacterial chromosome adjacent to a SCCmec IV element. It has been suggested that the physical linkage between ACME and SCCmec type IV leads to enhanced fitness by coselecting for beta-lactam resistance and possibly other multidrug resistant determinants (9,12,29). In contrast to the findings in *S. aureus*, and in line with Miragaia *et al.* (11), we found no association between ACME and SCCmec type IV in *S. epidermidis*. Furthermore, we actually observed a significant lower rate of methicillin and gentamicin resistance among ACME-positive versus ACME-negative *S. epidermidis* isolates. The recombinases used for integration of ACME and SCCmec into the bacterial chromosome are able to integrate these two genetic elements independently (9,11), and different selection mechanisms may apply for *S. aureus* and *S. epidermidis*. Avoidance of carrying large genetic elements encoding antibiotic resistance or biofilm production may theoretically increase biologic fitness and thereby improve the capacity of colonizing *S. epidermidis* strains to grow and survive within a host.

There are limitations to our study. The isolates included are from a single Norwegian neonatal unit. Thus, it may be difficult to generalize our findings to *S. epidermidis* strains in other countries. Distinguishing between “true” *S. epidermidis* sepsis and blood culture contamination is also notoriously difficult, especially in neonates. However, our definition of neonatal *S. epidermidis* sepsis is in line with others (1), and it includes clinical signs of sepsis and a significant increase in host inflammatory response judged by CRP. We studied selected parts of the host inflammatory response *in vitro* and some parameters of interest could have been missed. However, we argue that our fairly broad approach cover central parts of host inflammatory response. Other investigators have used isogenic wild-type/ACME-deletion mutant pair for comparison of virulence (9,26). In the human whole-blood model, we used eight ACME-positive and eight ACME-negative *S. epidermidis* blood culture isolates matched for different putative virulence factors, but it is possible that other confounding virulence factors could have influenced the inflammatory response. The concentration of *S. epidermidis* used in our whole-blood model is high. However, this concentration is similar to lethal and sublethal doses of *Staphylococci* used in primate models of sepsis (30,31). During sepsis, bacteria often demonstrate massive growth within the first 12–24 h after entering the blood stream (32). The natural bactericidal activity of blood then subsequently reduces the viability of organisms that can be recovered from blood cultures (33). Quantitative neonatal blood cultures often reveal CoNS concentration around 10^3 CFU/mL. However, sepsis studies assessing total bacterial DNA load have shown that the difference between dead and live bacteria may be three to four orders of magnitude, and that both dead and live bacteria induce the innate immune system (32,34). We assessed the inflammatory response during a short 3-h period and, thus, argue that the bacterial concentration used in our experiments was adequate.

CONCLUSIONS

We conclude that ACME in *S. epidermidis* from neonates was associated with a lower prevalence of antibiotic resistance and biofilm production compared with the ACME-negative isolates. ACME was more commonly found in *S. epidermidis* blood culture isolates considered as contaminants and isolates obtained from mature babies. We found neither *in vivo* nor *in vitro* association between ACME in *S. epidermidis* and increased host inflammatory response. We suggest that the presence of ACME in *S. epidermidis* does not seem to lead to those isolates being more harmful.

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