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Published in:
Journal of Antimicrobial Chemotherapy

DOI:
10.1093/jac/dkx481

Publication date:
2018

Document version
Publisher's PDF, also known as Version of record

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Citation for published version (APA):
Edslev, S. M., Clausen, M. L., Agner, T., Stegger, M., & Andersen, P. S. (2018). Genomic analysis reveals different mechanisms of fusidic acid resistance in Staphylococcus aureus from Danish atopic dermatitis patients. Journal of Antimicrobial Chemotherapy, 73(4), 856-861. https://doi.org/10.1093/jac/dkx481
Genomic analysis reveals different mechanisms of fusidic acid resistance in *Staphylococcus aureus* from Danish atopic dermatitis patients

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Received 19 September 2017; returned 9 November 2017; revised 14 November 2017; accepted 21 November 2017

**Background:** *Staphylococcus aureus* skin colonization is common in patients with atopic dermatitis (AD) and is associated with risk of skin infections. AD patients therefore often receive antibiotic treatments, including topical treatment with fusidic acid, which have been associated with resistance development.

**Objectives:** To examine the prevalence of antibiotic resistance in *S. aureus* isolated from Danish AD patients, with a primary focus on fusidic acid resistance and the genetic mechanisms that underlie it.

**Methods:** One hundred and thirty-eight *S. aureus* isolates collected from lesional skin (n = 54), non-lesional skin (n = 27) and anterior nares (n = 57) from 71 adult AD patients were included in the study. Isolates were tested for susceptibility to 17 selected antibiotics. *S. aureus* whole-genome sequences were used to examine the genetic determinants of fusidic acid resistance (*fusa* or *fusE* mutations or carriage of *fusB* or *fusC* genes).

**Results:** One hundred and nine isolates (79%) were resistant to at least one of the tested antibiotics, with the most prevalent resistances being to penicillin (55%), fusidic acid (41%) and erythromycin (11%). The primary genetic mechanisms of fusidic acid resistance were carriage of *fusC* (57%) or mutations in *fusa* (38%). The most prevalent *S. aureus* lineage was ST1 (23%). All ST1 isolates carried *fusC*.

**Conclusions:** *S. aureus* fusidic acid resistance, caused by either *fusa* mutations or *fusC* gene carriage, is a major concern among AD patients. Resistant *S. aureus* might spread from the patients to the community, indicating the need to reduce the use of fusidic acid in the treatment of AD.

**Introduction**

Atopic dermatitis (AD) is a common inflammatory skin disease that affects 10%–20% of children and 2%–10% of adults worldwide. The disease pathogenesis is multifactorial and includes impaired skin barrier function and an imbalance of the immune system with increased Th2-mediated pro-inflammatory responses. AD patients are more often colonized with *Staphylococcus aureus* compared with non-AD individuals [OR 19.7 for lesional skin (LS), 7.8 for non-lesional skin (NLS) and 4.5 for anterior nares]. This leads to increased disease severity, risk of skin infections and prescription of antibiotics. In many countries, including Denmark, topical treatment with fusidic acid ointment, often in combination with corticosteroids, has been one of the preferred choices of treatment. Fusidic acid is a narrow-spectrum antibiotic used against *S. aureus*. It inhibits bacterial growth by interacting with elongation factor G (EF-G) upon ribosome binding, thereby stalling protein synthesis. Though fusidic acid is known to select for resistance in *S. aureus*, the resistance prevalence among clinical isolates is generally considered to be low. However, an increasing prevalence of fusidic acid-resistant *S. aureus*, especially isolates from skin and soft tissue infections, has been observed in many European countries since the mid-1990s. Two different mechanisms of fusidic acid resistance have been identified in clinical *S. aureus* strains: (i) mutations in the gene encoding the drug target EF-G (*fusa*) or the gene encoding ribosome protein L6 (*fusE*), which interacts with EF-G upon ribosome binding; and (ii) horizontal acquisition of genes (*fusB* or *fusC*), encoding proteins that block the binding of fusidic acid to EF-G.

The *fusB* gene is carried on plasmids or a genomic island on the chromosome and the *fusC* resistance mechanisms of fusidic acid resistance have been identified in clinical *S. aureus* strains: (i) mutations in the gene encoding the drug target EF-G (*fusa*) or the gene encoding ribosome protein L6 (*fusE*), which interacts with EF-G upon ribosome binding; and (ii) horizontal acquisition of genes (*fusB* or *fusC*), encoding proteins that block the binding of fusidic acid to EF-G.

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gene is found integrated in a staphylococcal cassette chromosome (SCC) element. Several studies have examined the prevalence of fusidic acid resistance among S. aureus from AD patients, reporting varying resistance frequencies (3%–33%) depending on the country of origin. However, to our knowledge, no study has yet examined the genetic mechanisms behind fusidic acid resistance in S. aureus from AD patients.

The aim of this study was to determine the antibiotic resistance prevalence, with a primary focus on fusidic acid resistance, among skin and nasal S. aureus isolates from adult AD patients in Denmark. Furthermore, we aimed to identify and characterize the underlying genetics of fusidic acid resistance using WGS data.

Patients and methods

Patients

This study includes 71 AD patients from the outpatient clinic of the Department of Dermatology at Bispebjerg University Hospital (Denmark), who were all colonized with S. aureus in at least one of three sites: LS, NLS and anterior nares. The study population is part of a larger cohort (n=101), which has been described previously. Patients were included in the study during the period 2013–15. Inclusion criteria were age ≥18 years and presence of AD according to UK criteria. Exclusion criteria were pregnancy, breastfeeding and UV therapy within the last 2 months of sampling. Upon inclusion, AD disease severity was assessed using the Scoring Atopic Dermatitis (SCORAD) index and a blood sample was collected for subgroup testing for filaggrin gene (FLG) mutations that are common among Caucasians (R501X, 2282del4 and R2447X).

Bacterial isolate collection

Bacterial swabs were taken from LS, NLS and anterior nares using e-Swabs (Copan, Brescia, Italy). Skin samples were taken by rubbing the swab against the skin for 30 s and the nasal samples were taken by rotating the swab three times in one of the anterior nares. S. aureus was identified by plating samples on selective plates (chromID S. aureus plates, bioMérieux, Marcy-l’Étoile, France) and a single isolate was collected from each sample for further analysis.

Antibiotic susceptibility testing

Disc diffusion was performed using the Thermo Scientific Sensititre system (Trek Diagnostic Systems, Waltham, MA, USA) according to the methodology and breakpoints of EUCAST for the following antibiotics: penicillin, fusidic acid, erythromycin, clindamycin, tetracycline, kanamycin, rifampicin, norfloxacin, mupirocin, cefoxitin, trimethoprim/sulfamethoxazole, cefotibprole, ceftaroline, daptomycin, gentamicin, linezolid and vancomycin.

DNA extraction and WGS

DNA was extracted from isolates using a QiaGen DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), with a customized pretreatment with lysozyme and lysostaphin for a 30 min incubation period at 37°C. Whole-genome sequences (2×250 paired-end sequences) were obtained using a Nextera XT DNA Library Preparation Kit and a MiSeq sequencer (Illumina Inc., San Diego, CA, USA). The accession numbers for the Illumina sequences for all isolates are available at the European Nucleotide Archive (ENA) under the project identification number PRJEB18560.

| Table 1. Demographic and clinical characteristics of the AD study population (n=71) |
|-----------------------------|-------------------------------|
| Variable                  | Number of patients (%)       |
| Gender                     |                               |
| male                      | 33 (46.5)                     |
| female                    | 38 (53.5)                     |
| Age (years)               |                               |
| mean (range)              | 36.7 (18–77)                  |
| SCORAD                     |                               |
| mean (+ SD)               | 35.0 (+ 15.3)                 |
| FLG status                 |                               |
| WT                         | 38 (53.5)                     |
| mutations                  | 26 (36.6)                     |
| unknown                    | 7 (9.9)                       |
| Systemic antibiotic       |                               |
| treatment¹                 |                               |
| yes                        | 15 (21.1)                     |
| no                         | 56 (78.9)                     |
| Topical fusidic acid       |                               |
| treatment¹                 |                               |
| yes                        | 7 (9.9)                       |
| no                         | 64 (90.1)                     |
| Topical corticosteroid     |                               |
| treatment¹                 |                               |
| yes                        | 50 (70.4)                     |
| no                         | 18 (25.4)                     |
| unknown                    | 3 (4.2)                       |
| Systemic treatment         |                               |
| for AD⁰ᵇ                   |                               |
| yes                        | 22 (31.0)                     |
| no                         | 49 (69.0)                     |

Note: Within 3 months prior to sample collection.

Genetic typing and SNP analysis

Sequences were de novo assembled using SPAdes and contigs were used for MLST the isolates in silico using MLST by T. Seemann (https://github.com/tseemann/mlst.git). Clonal complex (CC) types were assigned from the STs using the algorithm eBURST v3 (http://eburst.mlst.net/). spa types were obtained by PCR and Sanger sequencing.

SNPs in the core genome were detected using the Northern Arizona SNP pipeline (NASP) by aligning de novo assembled contigs. A random selected isolate from the study population was used as a reference. The genetic diversity among isolates was examined by measuring the SNP distance between isolates in Geneious v10.0.9 (Biomatters Ltd, Auckland, New Zealand).

The genetic diversity among ST1 isolates was compared with 48 randomly selected bacteraemia ST1 MSSA isolates (isolation years 2011–14) from the Danish National Staphylococcus Surveillance Laboratory (Statens Serum Institut, Copenhagen, Denmark). SNPs in the core genome were identified by aligning assembled contigs from all isolates against the genome of the ST1 isolate MSSA176 (GenBank accession number BX571857) using NASP. A maximum-likelihood phylogeny was created in PhyML v.3.0, using Smart Model Selection with the Bayesian information criterion, 100 bootstrap replicates, nearest neighbour interchange and subtree pruning and grafting searches for optimal tree structure. The tree was visualized and annotated in iTOL (https://itol.embl.de/).

Identification of antibiotic resistance genes

Acquired antibiotic resistance genes, including fusB, were detected using the application ResFinder v2.1 (settings: minimum identity 90%; minimum coverage 90%). The MSSA176 genome was used as a reference for detection of the fusC gene and the SCC176 element. Identification was based on a BLAST search as well as mapping of reads against the reference. For 12 samples, which had <10-fold coverage of the fusC gene, a fusC-specific PCR was performed using the primers and procedure described previously.
in order to confirm the WGS results. Resistance-associated mutations in fusa and fusE were identified by aligning the gene sequences from each isolate to the WT gene sequences using MSSA_{76} as a reference (fusa, SAS0505; fusE, SAS2126). All genetic analyses were performed using CLC bio’s Genomics Workbench v8.5.1 (Qiagen, Aarhus, Denmark) if nothing else is stated.

**In silico identification of a novel fusB plasmid**

In order to identify the location of the fusB gene in three highly similar isolates (7–75 SNPs separating them) originating from a single patient, a BLAST search using the largest de novo assembled contig containing the resistance gene was performed against NCBI’s nucleotide sequence database, with the best hit being S. aureus plasmid pSA564 (NCBI accession number CP010891). The contig coverage was >4-fold higher than the average coverage of the chromosome, indicating a plasmid origin. The contig was circularized after removal of a 127 bp repetitive region and the splicing was verified by reference assembly using the original whole-genome sequence data. An alignment of the novel plasmid and the closest NCBI hit, pSA564, was performed using MAUVE as implemented in Geneious v10.0.9 to identify plasmid differences. The assembled fusB plasmid was annotated using Prokka v1.1133 and submitted to NCBI’s nucleotide sequence database under the accession number MF622050.

**Ethics**

The study was approved by the local Ethics Committee (project number 1-2014-039) and the Danish Data Protection Agency (project number 01767 BBH- 2012-019). Patients gave informed consent to participate in the study.

**Statistics**

The non-parametric Mann–Whitney U-test was used to test for differences in the pairwise SNP distance between groups. Statistical analysis was performed in R v3.3.2 (R Foundation for Statistical Computing, Vienna, Austria).

**Results**

**Isolate collection**

One hundred and thirty-eight S. aureus isolates collected from LS (n = 54), NLS (n = 27) or anterior nares (n = 57) from 71 adult AD patients were included in the study. The isolate collection was clonally diverse and consisted of 21 different STs, which could be grouped into 17 different CCs and a singleton (Table S1, available as Supplementary data at JAC Online). The most prevalent CC types were CC1 (n = 32; ST1), CC15 (n = 27; ST15 and STS82) and CC45 (n = 19; ST45 and ST508). Fifty-one different spa types were identified (Table S2). For 42 of 46 patients (91%) with more than one S. aureus-positive swab, the same clonal S. aureus type was isolated from the different sample sites. Pairwise SNP distance analysis showed that S. aureus isolates from the same patient (mean SNP distance: 1677) were significantly more similar compared with isolates collected from different patients (mean SNP distance: 27045) (P < 0.001) (Table S3).

In order to examine whether the high frequency of ST1 isolates was due to local transmission between AD patients at the outpatient clinic at the hospital, the genetic similarity between ST1 isolates (n = 32) from the AD patients and ST1 MSSA isolates (n = 48) from Danish bacteraemia patients was investigated in a phylogenetic analysis. The isolates from AD patients were dispersed throughout the phylogeny between different bacteraemia isolates (Figure S1). Also, a pairwise SNP distance analysis showed that S. aureus isolates from distinct AD patients (mean SNP distance: 291) were not genetically more similar compared with S. aureus from bacteraemia patients (mean SNP distance: 299) (P = 0.4) (Table S4).

**Antibiotic resistance prevalences**

One hundred and nine of the 138 S. aureus isolates (79%) were resistant to at least one of the tested antibiotics. The most prevalent types of resistance were resistance to penicillin (55%), fusidic acid (41%) and erythromycin (11%) (Table 2). Only a few S. aureus isolates were resistant to any other antibiotic tested and all were susceptible to mupirocin. Resistance genes identified in the resistant S. aureus isolates are given in Table S5.

At the patient level, 55 out of the 71 AD patients (78%) carried an antibiotic-resistant strain in at least one of the sampled sites and 27 patients (38%) were colonized with a fusidic acid-resistant strain. The overall antibiotic susceptibility profile across sampling sites (LS, NLS and anterior nares) was identical in 35 out of 54 LS patients (65%) and 27 patients (38%) were colonized with a fusidic acid-resistant strain. The overall antibiotic susceptibility profile across sampling sites (LS, NLS and anterior nares) was identical in 35 out of 46 patients (76%) with more than one positive S. aureus swab. For the remaining 24%, there was no correlation of pattern to the site of sampling.

**Genetic determinants of fusidic acid resistance**

Carriage of one or more of the known genetic determinants of S. aureus fusidic acid resistance was identified in all the fusidic acid-resistant isolates (Table 3). The predominant types of resistance were carriage of fusc (57%) and point mutations in fusa (38%). Among nasal isolates, the prevalence of fusc gene carriage (65%) was remarkably higher than the prevalence of fusa mutations (30%), but no corresponding differences were observed among skin-colonizing isolates (LS and NLS). The fusa mutations were identified in five different STs, whereas the fusc gene was only identified in ST1 isolates (Figure S2).

Eight different fusa mutations causing amino acid substitutions in EF-G were detected in the resistant isolates (Table S6). A single isolate contained four different mutations, of which two (V90I and L641S) have been reported previously to be associated with fusidic acid resistance in S. aureus. It is unknown whether the last two
mutations (P286T and P406T) are associated with resistance to fusidic acid.

Three isolates, all from the same patient, had both a missense mutation in fusA (V86A) and a nonsense mutation in fusE (Y168Stop) (Table S6). As neither of the two mutations has been described previously, it is uncertain whether both mutations, or only one of them, are causing the observed resistance to fusidic acid.

The fusB gene was identified in three isolates, all originating from a single patient. The gene was localized on a novel 28,623 bp plasmid, which was very similar to S. aureus plasmid pSA564 (NCBI accession number CP010891), but contained an additional region (3173 bp) in which the fusB gene was integrated. The plasmid was named pSA564-fus and submitted to NCBI's nucleotide sequence database (accession number MF622050).

The fusC gene was integrated in an SCC476 element and was identified in all ST1 isolates in this study (n = 32).

### Topical fusidic acid treatment in relation to resistance

Seven of the 71 AD patients had been treated topically with fusidic acid within 3 months prior to the sample collection (Table 1) and 5 of these had been treated within the last week. Fusidic acid-resistant S. aureus was isolated from six of these patients (86%), whereas 21 out of 64 (33%) of the non-treated patients were colonized with fusidic acid-resistant S. aureus. At the bacterial level, 17 out of 18 isolates (94%) collected from patients who had received topical treatment were tested to be resistant to fusidic acid. In comparison, 39 out of 120 (33%) of the isolates collected from non-treated patients were resistant.

Eleven of the 18 isolates from patients recently treated with fusidic acid carried the fusC gene, 3 had fusA mutations and 3 had a combination of fusA and fusE mutations.

### Discussion

To our knowledge, the present study is the first to identify the genetic mechanisms underlying fusidic acid resistance in S. aureus isolated from AD patients. The predominant genetic determinants were carriage of fusC in an SCC476 element and fusA point mutations. There were no major differences in the prevalence of fusC carriage and fusA mutations among skin-colonizing isolates, but the prevalence of fusC (65%) was notably higher than the prevalence of fusA mutations (30%) in nasal isolates. Mutation in fusA is known to have a high fitness cost for S. aureus.10 This might explain why fewer nasal isolates carried the mutations, as topical fusidic acid preparations are only applied to the skin and S. aureus colonizing the nares have thus no advantage in harbouring fusA mutations, contrary to skin-colonizing bacteria. In comparison, carriage of the fusC gene has been shown not to impose a significant fitness cost in S. aureus.15 Due to the high fitness cost related to fusA mutations, it might be that only a minor subset of S. aureus isolates in a host contain such mutations. It is thus a limitation of the present study that only one isolate was selected from each of the sampling sites, as the prevalence of S. aureus with fusA mutation might have been underestimated.

Forty-one percent of S. aureus isolates from the Danish AD patients were resistant to fusidic acid. In comparison, 16% of all S. aureus from bacteraemia cases in Denmark in 2015 were resistant to this antibiotic15 and in a study of asymptomatic nasal carriers (n = 6814) in 2010–11 from nine European countries only 2.8% of MSSA isolates were fusidic acid resistant.36 It is not surprising that the prevalence of resistant isolates was higher among patients who had been treated topically with fusidic acid within the last 3 months compared with those who had not been treated. However, the group of patients who had received topical fusidic acid treatment was too small (n = 7) for any conclusions to be drawn.

Another study in the UK recently examined the effect of antibiotic treatments on S. aureus resistance development in children with AD.18 They found that topical fusidic acid treatment was associated with a significant increase in the resistance prevalence 2 weeks after the treatment, but not after 3 months, indicating that topical treatment only causes short-term resistance selection. However, they still found that the frequency of skin-colonizing S. aureus that were fusidic acid resistant was relatively high after the 3 month period (25%). Likewise, we found that 33% of S. aureus isolates from patients who had not been treated topically with fusidic acid in the past 3 months were resistant. This indicates that reducing the prevalence of resistant S. aureus in AD patients requires more restrictive regulation of the usage of topical fusidic acid. Such regulations were issued in Sweden in the early 2000s.

The national recommendation to reduce the consumption of fusidic acid ointments in Sweden was followed by a nationwide decrease in the prevalence of resistant S. aureus isolated from patients with skin infections.37

In addition to fusidic acid, the present study also evaluated S. aureus susceptibility to 16 other selected antibiotics. Of these, resistance prevalences >10% were only observed for penicillin and erythromycin. However, the percentage of isolates being penicillin resistant (55%) was lower than the resistance prevalence observed among S. aureus from Danish bacteraemia patients (71%).15 Also, there was no significant difference in the prevalence of erythromycin resistance between AD isolates (11%) and bacteraemia isolates (7%). Thus, resistance to antibiotics other than fusidic acid is not an issue restricted to AD.

The frequency of ST1 S. aureus in this study (23%) was notably higher than the frequency among Danish asymptomatic nasal colonization.
S. aureus carriers (7.7%) and Danish S. aureus bacteremia patients (8.6%). A likely explanation of the higher selection of ST1 S. aureus colonizing AD patients could be the presence of fusC, which was identified in all the studied ST1 isolates. Frequent treatments with fusidic acid among AD patients could lead to clonal selection and expansion of fusC-positive ST1 S. aureus in this patient group. Carriage of fusidic acid resistance genes has previously been shown to have a significant influence on the dissemination of S. aureus clones. WGS data from the Danish National Staphylococcus Surveillance Laboratory have shown that ~50% of sequenced ST1 MRSA (n = 79) isolated from either infections or asymptomatic carriers in 2007–14 also carried the fusC gene (data not shown). If these ST1 MRSA strains were to be introduced into the AD patient population, fusidic acid treatment could lead to MRSA selection and spread, as has been the case in New Zealand.

Factors other than fusC carriage, such as clone-specific virulence genes or host genetics, could also have an influence on the selection of ST1 S. aureus in AD. In accordance, we have recently shown a possible association between ST1 S. aureus colonization and mutations in FLG, encoding the human skin barrier protein filaggrin, in AD patients. The high frequency of ST1 S. aureus colonization in the AD patient group could possibly also be caused by local transmission between patients at the outpatient clinic. However, ST1 isolates from different AD patients were not genetically more similar than ST1 isolates from Danish bacteremia patients, thus excluding a local transmission at the clinic.

In summary, we found here that a high proportion of S. aureus colonizing skin and nares in Danish AD patients are resistant to fusidic acid and that the predominating genetic basis of resistance was carriage of fusC or mutations in fusA. The fusC gene was identified in all ST1 S. aureus isolates and is likely a major driver for the success of the ST1 S. aureus clone in the AD population. The skin and nares of AD patients can thus act as a reservoir for fusidic acid-resistant S. aureus that can spread to the community. This supports the argument that usage of fusidic acid preparations in the treatment of AD should be restricted.

Acknowledgements
Thanks to Elvira Chapka, Lone Ryste Hansen Kildevang, Ditte Marie Brix and Alexandra Medina Madsen (Statens Serum Institut) for technical assistance. Also, thanks to Anders Rhod Larsen, Head of the Danish National Staphylococcus Surveillance Laboratory (Statens Serum Institut), for sharing sequence data and information.

Funding
This study was supported by internal funding.

Transparency declarations
None to declare.

Supplementary data
Tables S1 to S6 and Figures S1 and S2 are available as Supplementary data at JAC Online.

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