International Journal of Medical Microbiology 309 (2019) 283–287

Contents lists available at ScienceDirect

International Journal of Medical Microbiology

journal homepage: www.elsevier.com/locate/ijmm

The prevalence of *Staphylococcus aureus* with mucoid phenotype in the airways of patients with cystic fibrosis—A prospective study

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ARTICLE INFO

Keywords:
Cystic fibrosis
S. aureus
Mucoid phenotype
Biofilm
Ica

ABSTRACT

Background: *Staphylococcus aureus* is one of the most frequently isolated pathogens in the respiratory tract of CF patients. Recently, we characterized peculiar mucoid *S. aureus* isolates, which are excessive biofilm formers and which carried a 5bp-deletion within the intergenic region of the *ica* operon. In this prospective study, we determined the prevalence of mucoid *S. aureus*-isolates in the airways of CF-patients during a 3-months period.

Methods: We analyzed specimens (sputa, throat swabs) from 81 CF patients who attended two CF centers in Münster, Germany. Ten *S. aureus* isolates were randomly picked from every *S. aureus*-positive airway specimen and evaluated for mucoidy using Congo Red agar and phenotypic tests. Mucoid isolates were characterized by *spa* sequence typing, biofilm production and sequencing of the intergenic region of the *ica* operon to screen for the 5bp-deletion.

Results: In 7 of 81 examined patients (8.6%), we detected mucoid *S. aureus* phenotypes (37 out of 1050 isolates; 3.5%). Twenty-five mucoid isolates carried the 5bp-deletion. Mucoid isolates produced excessive biofilm and were significantly more resistant to certain antibiotics.

Conclusions: In our prospective study, mucoid *S. aureus* was present in 8.6% of *S. aureus*–positive CF-patients. In 6 of 7 patients, mucoid isolates carried the 5bp-deletion, indicating that also other so far not identified mechanisms cause excessive biofilm formation. Further studies are necessary to ascertain the clinical impact of mucoid *S. aureus* phenotypes on the severity of the CF disease.

1. Introduction

Respiratory failure resulting from chronic and recurrent bacterial infection and inflammation remains to be the leading cause of death for those suffering from cystic fibrosis (CF) (MacKenzie et al., 2014). With an overall prevalence of approximately 70%, *Staphylococcus aureus* is not only one of the first pathogens to colonize and infect the respiratory tract of CF-patients, but it is also the most frequently isolated bacterium from CF-airways (Cystic Fibrosis Foundation, 2016). *S. aureus* is known to persist over many years and can be present for decades within the airways of CF-patients leading to decreased lung function (Junge et al., 2016; Schwerdt et al., 2018). Recently, Schwartbeck, Birtel et al. described hyper-biofilm producing *S. aureus* isolates with a mucoid phenotype, which were recovered from respiratory specimens of CF-patients (Schwartbeck et al., 2016) and showed a constitutive expression of biofilm due to a 5bp-deletion in the intergenic region of the intercellular adhesion (*ica*) operon (Heilmann et al., 1996), which is responsible for a polysaccharide composed biofilm named polysaccharide intercellular adhesin (PIA). However, the prevalence of mucoid *S. aureus* isolates is yet unknown.

The aim of this prospective study was the determination of the prevalence of mucoid *S. aureus* isolates in the airways of CF-patients over a 3-months period in two CF-centers. Furthermore, we investigated the antibiotic susceptibility, biofilm formation as well as the genetic background of mucoid and normal isolates, and determined, if the mucoid isolates carried the recently described 5bp-deletion.

2. Methods

2.1. Study design

Included in our prospective study were all CF-patients with *S.*

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https://doi.org/10.1016/j.ijmm.2019.05.002
Received 1 February 2019; Received in revised form 10 April 2019; Accepted 10 May 2019

aureus-positive cultures in their respiratory specimens, who attended the CF-outpatient clinics of the University Hospital Münster or the Clemenshospital Münster, Germany, from beginning of March until the end of May 2017. Sputum or throat swabs were obtained during the visits and were sent to the central study laboratory of the Institute of Medical Microbiology, Münster, Germany. All samples were cultured according to standard procedures for CF airway cultures (Häfner et al., 2014; Podbielski et al., 2017). In case of *S. aureus* growth, 10 *S. aureus* isolates were randomly picked from agar plates and were subjected to identification by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometer (Szabados et al., 2010). Confirmed *S. aureus* isolates were plated on Columbia Blood Agar (CBA) and Congo Red Agar (CRA).

Non-CF-patients (n = 18) with *S. aureus* cultures from airways, bones or soft tissue served as a control group for the identification of isolates with a mucoid phenotype.

2.2. Analysis of phenotype

Phenotypical characterization of size, pigmentation, mucoidy, hemolysis and beta toxin production of colonies on CBA was assessed after 24 h and 48 h incubation at 37 °C. On CBA, isolates with a mucoid phenotype form characteristic chewing gum like structures when picking up the colonies with a loop (Fig. 1). On CRA, mucoid phenotypes grow with characteristic wrinkled dry colonies after 48 h (Schwartbeck et al., 2016). The phenotype observed on CBA served as decisive criteria to distinguish between mucoid and non-mucoid isolates in this study.

2.3. Antibiotic susceptibility

Antimicrobial susceptibility was determined by the disk diffusion method according to EUCAST guidelines (EUCAST, 2017). All isolates were tested for the following antibiotics: penicillin G (10 µg), oxacillin (30 µg), gentamicin (10 µg), amikacin (30 µg), levofloxacin (5 µg), clindamycin (10 µg), erythromycin (15 µg), vancomycin (30 µg), rifampin (5 µg) and trimethoprim-sulfamethoxazole (25 µg). *S. aureus* isolates with a normal phenotype were streaked on Müller-Hinton (MH) agar, while strains that presented a small colony variant (SCV) phenotype were tested on CBA since they fail to grow on MH agar in the absence of thymidine (Besier et al., 2008). For the analysis, for each patient all isolates with different phenotypes (on CBA or CRA) or different results in susceptibility testing were included.

Fig. 1. *S. aureus* with mucoid phenotype. *S. aureus* with a mucoid phenotype stick tightly to the agar plates. When trying to pick the colonies with a loop, they form characteristic chewing gum like structures.

2.4. Biofilm assay

In order to evaluate biofilm production, biofilm assays were performed using a static 96-well polystyrene biofilm microtiter plate assay as described recently (Schwartbeck et al., 2016). Absorbance of the crystal violet staining was detected at 655 nm using a microplate photometer (Bio-Rad, Hercules, CA, USA). Every *S. aureus* isolate was investigated in three independent experiments with 8 technical replicates per plate to assure correctness and reproducibility of the results. On each microtiter plate, two reference strains served as controls, strain *Staphylococcus carnosus* TM300 as the negative (Rosenstein et al., 2009) and *Staphylococcus epidermidis* RP62 as the positive control (Sadovskaya et al., 2005).

We tested all mucoid isolates for biofilm formation, which were identified earlier due to their unusual sticky phenotype on CBA. Additionally, we exemplarily tested one isolate with a non-mucoid phenotype from each of the patients carrying mucoid isolates. If non-mucoid isolates showed different *spa*-types, we included all isolates with different *spa*-types in our examinations. The amount of biofilm produced by the positive control was set to 100%.

2.5. Spa-typing

Spa-typing of all 10 randomly picked *S. aureus* isolates of the patients with mucoid phenotypes was performed as described recently (Kahl et al., 2005). *Spa*-types were assigned using the Ridom Staph Type software (Ridom© GmbH, Münster, Germany).

2.6. Molecular analysis of the intergenic region of the ica operon

Amplification and sequencing of the *icaR-icaA* intergenic region of all 10 randomly picked *S. aureus* isolates was performed as described previously [4], if at least one mucoid isolate was cultured. Briefly, genomic DNA was isolated from *S. aureus* isolates by using the QiAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer instructions. Amplification of the intergenic region was performed in a total volume of 20 µL using the *Phusion*-High-Fidelity Polymerase (New England Biolabs, Frankfurt, Germany) and the primer pair Ica-Repressor-for 5´-CAA-TATCGATTTGTATTGTCAACTTT-3´ and Ica-Repressor-rev 5´-GGTTG-TAAGCCATATGGTAATTGA-3´. PCR was accomplished using the Eppendorf Mastercycler EP Gradient (Eppendorf, Hamburg, Germany). Amplified products were purified with the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and subsequently sequenced by MWG Eurofins Operon (Karlsruhe, Germany). Sequence analysis was conducted using the software Clone Manager Suite 9 (Scientific & Educational Software, Durham, NC) and the reference sequence of *S. aureus* ATCC35556.

2.7. Statistical analysis

Data concerning patients' age, sex, BMI and lung function were recorded in Case Report Forms. For evaluation of the patients' lung function, spirometry reference values according to the Global Lung Function Initiative (GLI) were applied (Quanjer et al., 2012). Microbiological records for each patient were evaluated for co-infection with *Pseudomonas aeruginosa*. Statistical analysis was performed using IBM SPSS Statistics 24 software.

3. Results

3.1. Prevalence rates

Within a 3-months period, 153 CF-patients (age 1–56 years) attended the two certified CF-centers in Münster, Germany. During this period, we analyzed a total of 1050 *S. aureus* isolates, resulting from 105 visits of 81 different patients. Data from 14 patients were not

Table 1
Patients' characteristics.

	Patients with mucoid *S. aureus* isolates (n = 7)	Patients without mucoid *S. aureus* isolates (n = 74)	
Parameter			*p* value
Median age (IQR)[a]	29 (14.0–39.0)	17 (9.0–24.5)	0.103
No. (%) of males	5 (71.4)	36 (48.6)	0.249
Median BMI (IQR)	21 (20.0–25.0)	19 (17.0–21.0)	0.063
Median FEV1% (IQR)	78 (63.0–101.0)	75 (52.0–93.0)	0.641
Median VC% (IQR)	85 (83.0–116.0)	87 (69.5–100.25)	0.783
No. (%) of patients with *P. aeruginosa* coinfection in sputum and/or throat swab	4 (57.1)	24 (32.4)	0.189

[a] IQR = Interquartile range.

included in our analysis, because the specimens yielded less than 10 *S. aureus* colonies. From seven of 81 CF-patients (8.6%) with positive *S. aureus* cultures, *S. aureus* isolates with a mucoid phenotype were identified (Fig. 1). Clinical characteristics of these patients are listed in Table 1. The density of mucoid *S. aureus* isolates cultured from the specimens varied between one and eight out of ten isolates. In each of the patients that were found to carry mucoid *S. aureus* isolates, also non-mucoid isolates could be detected. Thirty-seven (3.5%) of all analyzed isolates (n = 1050) presented a mucoid phenotype on CBA (21 isolates cultured from sputa, 16 isolates from throat swabs). In the control group of non-CF-patients with *S. aureus* infection (lung, bone or soft tissue infection), no isolates with mucoid phenotype were discovered (0 of 180 isolates).

3.2. Patient characteristics

The median age was 29 years (IQR = 14–39 years) for CF-patients with mucoid isolates and 17 years (IQR = 9–24.5 years) for CF-patients without mucoid isolates (Table 1, $p = 0.103$). No significant differences were found in sex, BMI or lung function of patients with the different phenotypes. Furthermore, there were no differences concerning co-infection with *P. aeruginosa*.

3.3. Resistance pattern

Resistance pattern of non-mucoid versus mucoid *S. aureus* isolates (Table 2) differed significantly. Isolates with a mucoid phenotype were more resistant to gentamicin, amikacin, levofloxacin and rifampin ($p < 0.05$).

Table 2
Resistance pattern of mucoid/non-mucoid *S. aureus* to important anti-staphylococcal antibiotics.

	Mucoid *S. aureus* isolates (n = 9)	Non-mucoid *S. aureus* isolates (n = 156)	
Resistance to antibiotics[b] (%)			*p* value
Penicillin G	8 (88.9)	112 (71.8)	0.526
Oxacillin	1 (11.1)	10 (6.4)	0.583
Gentamicin	4 (44.4)	17 (10.9)	**0.003**
Amikacin	4 (44.4)	9 (5.8)	**< 0.001**
Levofloxacin	4 (44.4)	20 (12.8)	**0.032**
Clindamycin	2 (22.2)	38 (24.4)	0.884
Erythromycin	5 (55.6)	42 (26.9)	0.178
Vancomycin	0 (0.0)	0 (0.0)	(-)
Rifampin	4 (44.4)	11 (7.1)	**< 0.001**
Trimethoprim-Sulfamethoxazole	2 (22.2)	16 (10.3)	0.263

[b] total number of isolates that were resistant to the indicated antibiotics.

3.4. Biofilm formation

Biofilm formation of all mucoid isolates was quantitatively analyzed by a static 96-well biofilm assay. For statistical analysis, we compared one of the mucoid *S. aureus* isolates with a non-mucoid isolate obtained from the specimen of the same patient (Table 3). If possible, we tested mucoid and non-mucoid isolates of the same or a closely related *spa*-type. In 6 of 7 patients, mucoid isolates produced statistically significant higher amounts of biofilm than non-mucoid isolates (Fig. 2). *S. aureus* isolates with a mucoid phenotype from patient 01 did not form biofilm under the used conditions.

3.5. Genetic background

Mucoid and non-mucoid *S. aureus* isolates were of the same *spa*-type in four of seven patients (patient 02, 03, 04 and 05). In two patients, mucoid and non-mucoid isolates belonged to different *spa*-types (patients 01 and 07) as indicated by the very different repeats of *spa*-types. However, in patient 06, *spa*-types of mucoid and non-mucoid isolates were closely related as indicated by a small difference of the repeat region with a deletion of repeat 17 in the mucoid isolate (Table 3).

3.6. Investigation of the 5bp-deletion within the intergenic region of the ica operon

In the mucoid isolates from 6 of the 7 patients, we identified the 5bp-deletion within the intergenic region of the *ica* operon, which explained the mucoid phenotype as determined recently (Schwartbeck et al., 2016). However, despite a mucoid phenotype, in the mucoid isolates (n = 8) from patient 01, we were not able to detect the 5bp-deletion (Table 3) indicating another mechanism being responsible for mucoidy. In addition, in patient 03 (Table 3), there were mucoid and non-mucoid *S. aureus* isolates, which all presented the 5bp-deletion irrespective of their phenotype on CBA and CRA.

4. Discussion

Recently, we described a new phenotype of *S. aureus,* which was cultured from the airways of CF-patients, with unusual mucoid colonies that have not been reported so far from clinical specimens (Schwartbeck et al., 2016). The proper detection of *S. aureus* with a mucoid phenotype is hampered by the fact that mucoid isolates exhibit only a slightly varied phenotype on conventional standard media, which are used in routine microbiological diagnostics. The extraordinary phenotype often does not become evident until touching the colony with the isolation loop and thereby experiencing the stickiness of the colony. To estimate the prevalence of mucoid isolates, we performed a prospective pilot study in two certified CF centers in Münster. In this study, we used CRA agar to facilitate the identification of the mucoid phenotype (Brooks and Jefferson, 2014; Schwartbeck et al., 2016). Using this approach and by randomly picking 10 *S. aureus* colonies from every *S. aureus*-positive specimen, we identified 7 CF-patients (8.6% of *S. aureus*-positive CF-patients), who carried *S. aureus* with a mucoid phenotype.

Mucoid colony phenotypes with an increased production of biofilm have been described for various CF relevant pathogens such as *P. aeruginosa* (Pritt et al., 2007), *Burkholderia multivorans* (Silva et al., 2011) or *Achromobacter xylosoxidans* (Nielsen et al., 2016). It has been proven that mucoid *P. aeruginosa* plays an important role in CF lung disease progression (Li et al., 2005) and that its finding predicts pulmonary exacerbation and lung function decline (Mayer-Hamblett et al., 2014). In our study, we collected and recorded clinical data from microbiological and clinical records to examine whether similar trends can be observed for CF-patients with mucoid *S. aureus* isolates. Due to the small number of patients with mucoid isolates, we were not able to show a clinical impact of mucoid *S. aureus* on disease progression so far.

Table 3
Genetic background of mucoid and non-mucoid isolates.

No. of patient	No. of isolate	Phenotype on CBA[c]	*spa* type	Repeats[d]	5bp deletion
Pat. 01	M331	non-mucoid	t091	07-23-21-17-34-12-23-02-12-23	no
	M337	mucoid	t493	04-34-17-66-32-17-23-24	no
Pat. 02	M356	non-mucoid	t189	07-23-12-21-17-34	no
	M360	mucoid	t189	07-23-12-21-17-34	yes
Pat. 03	M361	non-mucoid	t002	26-23-17-34-17-20-17-12-17-16	yes
	M366	mucoid	t002	26-23-17-34-17-20-17-12-17-16	yes
Pat. 04	M451	non-mucoid	t267	07-23-12-21-17-34-34-34-33-34	no
	M456	mucoid	t267	07-23-12-21-17-34-34-34-33-34	yes
Pat. 05	M511	non-mucoid	t091	07-23-21-17-34-12-23-02-12-23	no
	M513	mucoid	t091	07-23-21-17-34-12-23-02-12-23	yes
Pat. 06	M571	non-mucoid	t003	26-17-20-17-12-17-17-16	no
	M575	mucoid	t893	26-20-17-12-17-17-16	yes
Pat. 07	M986	non-mucoid	t011	08-16-02-25-34-24-25	no
	M987	mucoid	t618	15-12-16-02-16-02-25-17-17-17-24	yes

[c] Columbia blood agar.
[d] repeats of *spa* types depending on the sequence of the variable number of tandem repeats as assigned by the Ridom *Spa* Software.

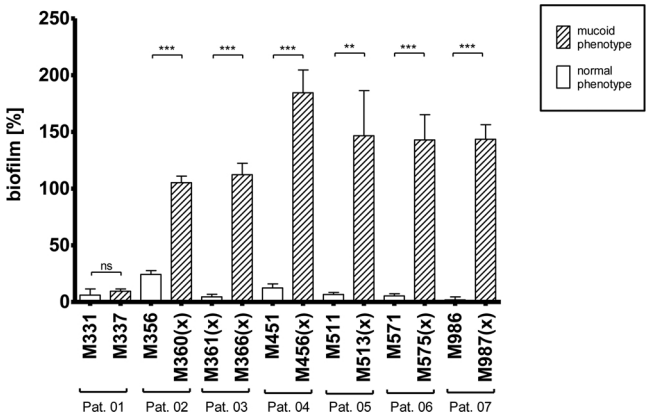

Fig. 2. Biofilm formation of paired mucoid and non-mucoid isolates from the same patient. Isolates, which carried the 5bp deletion are marked with (x). The biofilm produced by each isolate is depicted as the percentage of the biofilm produced by the positive control. Every *S. aureus* isolate was investigated in three independent experiments with 8 technical replicates per plate. (*** = Extremely significant (p value < 0.001); ** = Very significant (p value 0.001 to 0.01); * = Significant (p value 0.01 to 0.05); ns = Not significant (p value > 0.05)).

Therefore, prospective multicenter studies are necessary in order to follow a higher number of patients with mucoid isolates.

We did observe differences in age, albeit not statistically significant, between the patients with normal and mucoid *S. aureus*.

Mucoid *S. aureus* isolates revealed increased resistance to antibiotics in comparison to non-mucoid isolates, especially to gentamicin, amikacin, levofloxacin and rifampin. It has been shown in previous studies that bacteria, which live in biofilms develop certain antimicrobial resistance mechanisms and are therefore difficult to eradicate with conventional antimicrobial therapy (del Pozo and Patel, 2007). Interestingly, it has been shown in prior studies that particular antibiotics can induce increased biofilm formation. Schilcher et al. demonstrated that sub-inhibitory concentrations of clindamycin lead to an increased biofilm production by *S. aureus* USA300 LAC (Schilcher et al., 2016). However, there was no difference in resistance to clindamycin in mucoid and non-mucoid *S. aureus*. Furthermore, clindamycin is not often used to treat *S. aureus* airway infection in CF-patients.

Most mucoid isolates in our study produced great amounts of biofilm in the static microtitre plate assay. However, mucoid *S. aureus* isolates from one patient did not form any biofilm under the experimental conditions. Different underlying mechanisms in the formation of biofilm and a distinct composition of biofilm could explain these observations. Several studies report that biofilm can consist of a mixture of multiple components, e.g. PIA, teichoic acids, proteins and/or extracellular DNA (eDNA) (Kaplan et al., 2011; Sadovskaya et al., 2005). The isolates (n = 8) we observed for patient 01 grew with a pronounced sticky phenotype on CBA but did not form biofilm in the microtitre plate assay. Since those isolates neither grew in typical wrinkled colonies on CRA nor showed a characteristic mutation within the intergenic region of the *ica* operon, we assume that other components than PIA will be responsible for the mucoid phenotype in these 8 isolates. Further experiments are required in order to identify the biofilm matrix components of the mucoid isolates of patient 01.

In our study, the mucoid phenotype was not restricted to a certain *S. aureus* clone as indicated by the different *spa*-types. In four patients, mucoid and non-mucoid *S. aureus* isolates were of the same *spa*-type, indicating that they are closely related to each other. However, we also detected mucoid and non-mucoid isolates from the same specimen belonging to entirely different *spa*-types with different repeats (patients 01 and 07). In those cases, the acquisition of different *S. aureus* isolates at independent stages and hence a coexistence of strains of different genetic backgrounds in the patients' airways seem to be most likely.

In patient 06, the *spa*-type of the mucoid and non-mucoid isolate differed only by one repeat (Table 3). During long-term persistence within CF-airways, *S. aureus* isolates experience a variety of mutations in the bacterial genome, which can result in different *spa*-types, if the variable region of protein A is affected due to deletions or duplications of repeats or point-mutations (Kahl et al., 2005). When comparing the sequence of repeats in the variable *spa*-region of the mucoid (t893) and non-mucoid (t003) isolates from patient 06, a loss of only one repeat becomes evident. It can be assumed that the mucoid isolate of this patient descended from a non-mucoid strain during persistence as it has been shown earlier (Kahl et al., 2005; Schwartbeck et al., 2016).

All mucoid *S. aureus* isolates (n = 25) of 6 patients (patients 02 – 07) that carried the 5bp-deletion within the intergenic region of the *ica* operon showed a sticky phenotype on CBA and CRA. As previously shown (Jefferson et al., 2003, 2004; Schwartbeck et al., 2016), the 5bp-deletion within the *icaR-icaA* region was responsible for the unusual mucoid phenotype. Recently, a new regulator termed "repressor of biofilm" (*rob*) has been identified, which binds to exactly this region (Yu et al., 2017). In case of the 5bp-deletion, the repressor cannot bind to this region, which leads to a constitutive formation of biofilm as shown for most of the mucoid isolates.

The fact, that we also detected 5bp-deletions in non-mucoid isolates of patient 03 indicates the existence of additional compensatory mutations within the *ica* operon as we have shown recently (Schwartbeck et al., 2016). In the earlier publication, we identified compensatory mutations in *icaA, D* and *C*, leading to a normal phenotype on CBA and

CRA despite presence of the 5bp-deletion. Complementation with plasmids containing the respective intact wild-type *ica* genes restored mucoidy in all cases, corroborating that the compensatory mutations caused abrogation of increased biofilm formation (Schwartbeck et al., 2016). Therefore, we assume that these isolates of patient 03 also possess a secondary mutation in one of the four *ica* genes.

In 8 isolates with a mucoid phenotype of patient 01, we did not detect the 5bp-deletion. As the *ica* operon is regulated at different levels and by a multiplicity of regulators, which can interact directly or indirectly with the *ica* operon, it could be possible that mucoidy of these isolates is due to other mutations within the *ica* region. In the literature, the *ica* regulator (IcaR) and the teicoplanin-associated locus regulator (TcaR) are the best known negative regulators (repressors), which suppress the transcription of *icaADBC* (Jefferson et al., 2003, 2004; Yu et al., 2017). *in vitro*, hyper-expression of PIA by IcaR and/or TcaR of *S. epidermidis* and *S. aureus* mutants could be shown by targeted deletion mutagenesis (Conlon et al., 2002; Jefferson et al., 2004). To the best of our knowledge, clinical isolates with mutations in these two repressor genes have not been identified to date. To detect the mutations leading to mucoidy in these isolates will be the subject of further investigations.

5. Conclusions

In our prospective study, we were able to identify *S. aureus* isolates with a mucoid phenotype in almost 9% of all *S. aureus*-positive CF-patients. Mucoid isolates were mostly of the same or a closely related *spa*-type compared to the non-mucoid *S. aureus* isolated at the same time. Most but not all mucoid isolates carried the 5bp-deletion of the intergenic region of the *ica* operon, indicating that also other mutations are responsible for mucoidity. Mucoid isolates were more often resistant to antibiotics and mostly produced a significantly higher amount of biofilm compared to non-mucoid isolates. If there is an association of mucoid *S. aureus* and CF-disease progression, antibiotic treatment or co-infection with other pathogens could not be determined with the collected data due to the small number of patients and should be investigated in multicenter studies. Also, further studies are necessary to identify the underlying mechanism of mucoid isolates without 5bp-deletion.

Conflicts of interest

The authors do not have any conflict of interest.

Funding

This study was funded by the German Research Foundation, DFG (KA2249/5-1 to BCK) and by the Medical Faculty for thesis support (MedK to FEL).

Acknowledgements

We would like to thank Johanna Sobocinski, Barbara Grünastel and Lisa Krause for their support in isolating *S. aureus* from the complex microbiological cultures such as CF specimens and their enthusiasm for this study. We also thank Susanne Deiwick, Heike Rengbers and Julia Posdorfer for their help in carrying out biofilm assays, analyzing *spa*-types and investigating the antimicrobial susceptibility of the isolates.

References

Besier, S., Zander, J., Kahl, B.C., Kraiczy, P., Brade, V., Wichelhaus, T.A., 2008. The thymidine-dependent small-colony-variant phenotype is associated with hypermutability and antibiotic resistance in clinical *Staphylococcus aureus* isolates. Antimicrob. Agents Chemother. 52 (6), 2183–2189.

Brooks, J.L., Jefferson, K.K., 2014. Phase variation of poly-N-acetylglucosamine expression in Staphylococcus aureus. PLoS Pathog. 10 (7) e1004292.

Conlon, K.M., Humphreys, H., O'Gara, J.P., 2002. icaR encodes a transcriptional repressor involved in environmental regulation of *ica* operon expression and biofilm formation in *Staphylococcus epidermidis*. J. Bacteriol. 184 (16), 4400–4408.

Cystic Fibrosis Foundation, 2016. Cystic Fibrosis Foundation Patient Registry: 2015 Annual Data Report. Bethesda, Maryland. .

del Pozo, J.L., Patel, R., 2007. The challenge of treating biofilm-associated bacterial infections. Clin. Pharmacol. Ther. 82 (2), 204–209.

EUCAST, 2017. Clinical Breakpoint Tables for Interpretation of MICs and Zone Diameters: Version 7.0.

Häfner, L., Peters, G., Kahl, B.C., 2014. Assessment of microbiological diagnostic procedures for respiratory specimens from cystic fibrosis patients in German laboratories by use of a questionnaire. J. Clin. Microbiol. 52 (3), 977–979.

Heilmann, C., Schweitzer, O., Gerke, C., Vanittanakom, N., Mack, D., Götz, F., 1996. Molecular basis of intercellular adhesion in the biofilm-forming Staphylococcus epidermidis. Mol. Microbiol. 20 (5), 1083–1091.

Jefferson, K.K., Cramton, S.E., Götz, F., Pier, G.B., 2003. Identification of a 5-nucleotide sequence that controls expression of the *ica* locus in *Staphylococcus aureus* and characterization of the DNA-binding properties of IcaR. Mol. Microbiol. 48 (4), 889–899.

Jefferson, K.K., Pier, D.B., Goldmann, D.A., Pier, G.B., 2004. The Teicoplanin-Associated Locus Regulator (TcaR) and the Intercellular Adhesin Locus Regulator (IcaR) are transcriptional inhibitors of the *ica*Locus in Staphylococcus aureus. J. Bacteriol. 186 (8), 2449–2456.

Junge, S., Görlich, D., den Reijer, M., Wiedemann, B., Tümmler, B., Ellemunter, H., Dübbers, A., Küster, P., Ballmann, M., Koerner-Rettberg, C., Große-Onnebrink, J., Heuer, E., Sextro, W., Mainz, J.G., Hammermann, J., Riethmüller, J., Graepler-Mainka, U., Staab, D., Wollschläger, B., Szczepanski, R., Schuster, A., Tegtmeyer, F.-K., Sutharsan, S., Wald, A., Nofer, J.-R., van Wamel, W., Becker, K., Peters, G., Kahl, B.C., 2016. Factors associated with worse lung function in cystic fibrosis patients with persistent Staphylococcus aureus. PLoS One 11 (11) e0166220.

Kahl, B.C., Mellmann, A., Deiwick, S., Peters, G., Harmsen, D., 2005. Variation of the polymorphic region X of the *protein A* gene during persistent airway infection of cystic fibrosis patients reflects two independent mechanisms of genetic change in *Staphylococcus aureus*. J. Clin. Microbiol. 43 (1), 502–505.

Kaplan, J.B., Jabbouri, S., Sadovskaya, I., 2011. Extracellular DNA-dependent biofilm formation by *Staphylococcus epidermidis* RP62A in response to subminimal inhibitory concentrations of antibiotics. Res. Microbiol. 162 (5), 535–541.

Li, Z., Kosorok, M.R., Farrell, P.M., Laxova, A., West, S.E.H., Green, C.G., Collins, J., Rock, M.J., Splaingard, M.L., 2005. Longitudinal development of mucoid *Pseudomonas aeruginosa* infection and lung disease progression in children with cystic fibrosis. JAMA 293 (5), 581–588.

MacKenzie, T., Gifford, A.H., Sabadosa, K.A., Quinton, H.B., Knapp, E.A., Goss, C.H., Marshall, B.C., 2014. Longevity of patients with cystic fibrosis in 2000 to 2010 and beyond: survival analysis of the Cystic Fibrosis Foundation patient registry. Ann. Intern. Med. 161 (4), 233–241.

Mayer-Hamblett, N., Rosenfeld, M., Gibson, R.L., Ramsey, B.W., Kulasekara, H.D., Retsch-Bogart, G.Z., Morgan, W., Wolter, D.J., Pope, C.E., Houston, L.S., Kulasekara, B.R., Khan, U., Burns, J.L., Miller, S.I., Hoffman, L.R., 2014. *Pseudomonas aeruginosa* in vitro phenotypes distinguish cystic fibrosis infection stages and outcomes. Am. J. Respir. Crit. Care Med. 190 (3), 289–297.

Nielsen, S.M., Nørskov-Lauritsen, N., Bjarnsholt, T., Meyer, R.L., 2016. *Achromobacter* species isolated from cystic fibrosis patients reveal distinctly different biofilm morphotypes. Microorganisms 4 (3).

Podbielski, A., Herrmann, M., Kniehl, E., Mauch, H., Rüssmann, H., 2017. MiQ: Qualitätsstandards in der mikrobiologisch-infektiologischen Diagnostik: MiQ Grundwerk Heft. Elsevier; Urban & Fischer in Elsevier, München, pp. 1–29.

Pritt, B., O'Brien, L., Winn, W., 2007. Mucoid Pseudomonas in cystic fibrosis. Am. J. Clin. Pathol. 128 (1), 32–34.

Quanjer, P.H., Stanojevic, S., Cole, T.J., Baur, X., Hall, G.L., Culver, B.H., Enright, P.L., Hankinson, J.L., Ip, M.S.M., Zheng, J., Stocks, J., 2012. Multi-ethnic reference values for spirometry for the 3-95-yr age range: The global lung function 2012 equations. Eur. Respir. J. 40 (6), 1324–1343.

Rosenstein, R., Nerz, C., Biswas, L., Resch, A., Raddatz, G., Schuster, S.C., Götz, F., 2009. Genome analysis of the meat starter culture bacterium *Staphylococcus carnosus* TM300. Appl. Environ. Microbiol. 75 (3), 811–822.

Sadovskaya, I., Vinogradov, E., Flahaut, S., Kogan, G., Jabbouri, S., 2005. Extracellular carbohydrate-containing polymers of a model biofilm-producing strain, *Staphylococcus epidermidis* RP62A. Infect. Immun. 73 (5), 3007–3017.

Schilcher, K., Andreoni, F., Dengler Haunreiter, V., Seidl, K., Hasse, B., Zinkernagel, A.S., 2016. Modulation of *Staphylococcus aureus* biofilm matrix by subinhibitory concentrations of clindamycin. Antimicrob. Agents Chemother. 60 (10), 5957–5967.

Schwartbeck, B., Birtel, J., Treffon, J., Langhanki, L., Mellmann, A., Kale, D., Kahl, J., Hirschhausen, N., Neumann, C., Lee, J.C., Götz, F., Rohde, H., Henke, H., Küster, P., Peters, G., Kahl, B.C., 2016. Dynamic in vivo mutations within the *ica* operon during persistence of *Staphylococcus aureus* in the airways of cystic fibrosis patients. PLoS Pathog. 12 (11) e1006024.

Schwerdt, M., Neumann, C., Schwartbeck, B., Kampmeier, S., Herzog, S., Görlich, D., Dübbers, A., Große-Onnebrink, J., Kessler, C., Küster, P., Schültingkemper, H., Treffon, J., Peters, G., Kahl, B.C., 2018. *Staphylococcus aureus* in the airways of cystic fibrosis patients - a retrospective long-term study. IJMM 308 (6), 631–639.

Silva, I.N., Ferreira, A.S., Becker, J.D., Zlosnik, J.E.A., Speert, D.P., He, J., Mil-Homens, D., Moreira, L.M., 2011. Mucoid morphotype variation of *Burkholderia multivorans* during chronic cystic fibrosis lung infection is correlated with changes in metabolism, motility, biofilm formation and virulence. Microbiology (Reading, England) 157 (Pt 11), 3124–3137.

Szabados, F., Woloszyn, J., Richter, C., Kaase, M., Gatermann, S., 2010. Identification of molecularly defined *Staphylococcus aureus* strains using matrix-assisted laser desorption/ionization time of flight mass spectrometry and the Biotyper 2.0 database. J. Med. Microbiol. 59 (Pt 7), 787–790.

Yu, L., Hisatsune, J., Hayashi, I., Tatsukawa, N., Sato'o, Y., Mizumachi, E., Kato, F., Hirakawa, H., Pier, G.B., Sugai, M., 2017. A novel repressor of the *ica* locus discovered in clinically isolated super-biofilm-elaborating *Staphylococcus aureus*. mBio 8 (1).