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Extended *Staphylococcus aureus* persistence in cystic fibrosis is associated with bacterial adaptation

Nina Hirschhausen[a,1], Desiree Block[a,1], Irene Bianconi[c], Alessandra Bragonzi[c], Johannes Birtel[a,d], Jean C. Lee[d], Angelika Dübbers[e], Peter Küster[f], Janina Kahl[a], Georg Peters[a,b], Barbara C. Kahl[a,b,*]

[a] *Institute of Medical Microbiology, University Clinics Münster, 48149 Münster, Germany*
[b] *Interdisciplinary Centre for Clinical Research (IZKF), University of Münster, 48149 Münster, Germany*
[c] *Infections and Cystic Fibrosis Unit, Division of Immunology, Transplantation and Infectious Diseases, San Raffaele Scientific Institute, 20132 Milano, Italy*
[d] *Channing Laboratory, Brigham and Women's Hospital, Harvard Medical School, 02115 Boston, USA*
[e] *Department of Pediatrics, University Clinics Münster, 48149 Münster, Germany*
[f] *Department of Pediatrics, Clemenshospital Münster, 48153 Münster, Germany*

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ABSTRACT

Staphylococcus aureus often persists in the airways of cystic fibrosis (CF) patients. There is only limited knowledge about bacterial persistence in and adaptation to this new ecological environment. Therefore, we used *S. aureus* isolates from a unique strain collection, in which all *S. aureus* isolates recovered from CF patients from two CF centers were stored from more than 150 CF patients for more than a decade. *S. aureus* early and late isolates from 71 CF patients with long-term staphylococcal colonization of the airways (≥5 years) were preselected by genotyping of *agr* and *cap*. Identical pairs were subjected to *spa*-typing and MLST. *S. aureus* strain pairs of individual patients with the same or closely related *spa*-type and identical MLST were compared for adaptive changes in important phenotypic and virulence traits. The virulence of three *S. aureus* strain pairs (early and late isolates) was analyzed in a murine chronic pneumonia model. Strain pairs of 29 individual patients belonged to the same MLST and same or closely related *spa*-types. The mean persistence of the same clone of *S. aureus* in 29 CF patients was 8.25 years. Late compared to early isolates were altered in production of capsule (48%), hemolysis (45%), biofilm formation (41%), as well as antibiotic susceptibility (41%), cytotoxicity (34%), colony size (28%), and *spa*-type (17%). Adaptive changes positively correlated with the length of *S. aureus* persistence. For seven patients from whom the initial colonizing isolate was recovered, staphylococcal adaptation was most apparent, with capsule production being reduced in five of seven late isolates. In a mouse chronic pneumonia model, all tested isolates strongly induced chronic pneumonia with severe lesions in bronchi and pulmonary parenchyma. Adaptive changes in *S. aureus* accumulated with the length of persistence in the CF airways, but differed in patients infected with the same *S. aureus* clonal lineage indicating that individual host factors have an impact on adaptation.

Introduction

Cystic fibrosis (CF) is the most common autosomal recessive genetic disorder of the Caucasian population and is characterized by bronchial obstruction and impaired mucociliary clearance. CF patients experience chronic and recurrent bacterial infections that may result in respiratory insufficiency and early death (O'Sullivan and Freedman, 2009). *Staphylococcus aureus*, one of the earliest pathogens to colonize the CF airways, persists in the lungs for many years despite antibiotic intervention (Kahl et al., 2003; Stone and Saiman, 2007). Later, *S. aureus* is often replaced by *Pseudomonas aeruginosa*, the leading bacterial pathogen in CF. Nonetheless, ~50% of *P. aeruginosa*-infected CF patients still harbor *S. aureus* in their airways (Cystic Fibrosis Foundation, 2011; Viviani et al., 2012).

Whereas *S. aureus* asymptomatically colonizes the anterior nares and skin of healthy humans (Wertheim et al., 2005), it can also provoke a broad spectrum of community- and hospital-acquired infections, such as endocarditis, osteomyelitis, pneumonia, and sepsis (Lowy, 1998). The pathogen uses multiple strategies to

* Corresponding author at: Institute of Medical Microbiology, University Clinics Münster, Domagkstr. 10, 48149 Münster, Germany. Tel.: +49 251 8355358; fax: +49 251 8355350.
E-mail address: kahl@uni-muenster.de (B.C. Kahl).
[1] These two authors contributed equally to this work.

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escape host defenses and antibiotic treatment, *e.g.*, capsular polysaccharide (CP) production (O'Riordan and Lee, 2004), biofilm formation (Donlan and Costerton, 2002), diversification into heterogeneous morphotypes (Goerke et al., 2007), emergence of small colony variants (SCVs) (Kahl et al., 1998; Proctor et al., 2006), phage mobilization (Goerke and Wolz, 2004), and intracellular persistence (Garzoni and Kelley, 2009). Within the lungs of CF patients, *S. aureus* encounters the host immune response, antibiotics, interspecies competition, hypoxia and starvation, all of which trigger the process of adaptation (Hogardt and Heesemann, 2013; Goerke and Wolz, 2010). In contrast to the knowledge of *P. aeruginosa* adaptation to the CF airways (Hogardt and Heesemann, 2013), there is only limited information about the strategies that *S. aureus* exerts to adapt to this microenvironment (Goerke and Wolz, 2010).

We hypothesized that individual clones persist within the lungs of CF patients for extended periods and that persistence has an impact on bacterial phenotypic changes due to adaptation to the hostile niche. We determined the clonality of available early and late isolates of *S. aureus* from patients of two German CF centers, whose airways were colonized with *S. aureus* for ≥5 years. Indistinguishable *S. aureus* strain pairs (early and late isolates) from 29 patients were compared for their phenotypic and virulence characteristics, such as colony size, antibiotic susceptibility, *spa*-type, cytotoxicity for respiratory epithelial cells, and hemolysis, biofilm formation, and CP. Early and late *S. aureus* isolates from three CF patients with the longest persistence (>13 years) were selected for *in vivo* virulence analysis in a murine chronic pneumonia model.

Materials and methods

Selection and cultivation of bacterial strains

S. aureus isolates were chosen from a strain collection of *S. aureus* isolates, which were collected since 1994 from CF patients at the University Clinics and since 2001 of patients treated at the Clemenshospital in Münster, Germany. In both CF centers pediatric and adult patients are treated. Quantitative cultures of sputa were performed since 2001 according to standard procedures. Culture procedures for CF airway specimens were performed as described (Kahl et al., 1998). Briefly, morphologically different *S. aureus* colonies from primary cultures were sub-cultured to Columbia blood agar incubated at 37 °C and Schaedler agar incubated for 24 h at 37 °C under 5% CO_2. Resulting colonies were compared for size, hemolysis and pigmentation. All different morphotypes were frozen at −80 °C in 10% glycerol before further characterization.

Clinical data

Information about the *S. aureus* colonization status prior to the beginning of our retrospective study was retrieved from the patient's clinical records. Patient co-infection with *P. aeruginosa* was determined from the microbiology laboratory database.

Genotyping

Agr- and *cap*-typing were performed by multiplex PCR (Goerke et al., 2005). *Spa*-typing and MLST were performed as described previously (Harmsen et al., 2003; Enright et al., 2000; Kahl et al., 2005). In brief, for amplification of the seven *S. aureus* MLST housekeeping genes (*arc*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, *yqiL*), a PCR was performed using PuRe Taq Ready-To-Go PCR Beads (Amersham Biosciences, Freiburg, Germany). The PCR product was purified by an enzymatic method using exonuclease I (New England Biolabs GmbH, Frankfurt-Hoechst, Germany) and shrimp alkaline phosphatase (Amersham Biosciences). The amplicons were sequenced using the ABI Prism BigDye Terminator v3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Darmstadt, Germany). The sequencing products were purified with MultiScreen HV plates (Millipore, Schwalbach, Germany) loaded with Sephadex G50 Superfine columns (Amersham Biosciences) according to the instructions of the manufacturer (Millipore Tech Note TN053) and were then prepared for running on the ABI 3100 Avant Genetic Analyzer in accordance with the instructions of the manufacturer (Applied Biosystems). The software Ridom TraceEditPro™ (Ridom GmbH, Würzburg, Germany) was used for sequence analysis. The alleles and the sequence types (STs) were assigned using the *S. aureus* MLST database (http://saureus.mlst.net/).

Phenotypic characterization

S. aureus colony size was judged macroscopically on primary subcultures after thawing on Columbia blood agar. Colonies ≥10-fold smaller than typical *S. aureus* colonies, which have a mean size of 1.45 mm as measured for all investigated isolates by the colony counter Biocount Pro-beta (BIO-SYS GmbH, Karben, Germany), were designated SCVs.

Mice

Male C57BL/6NCrlBR mice (20–22 g, 6–8 weeks of age) were obtained from Charles River Laboratories, Italy. Infection with *S. aureus* was adapted from a model established for *P. aeruginosa* (Bragonzi et al., 2009). Animal studies were conducted according to protocols approved by the Institutional Animal Care and Use Committee of the San Raffaele Scientific Institute, Milan, Italy, in accordance with the Italian Ministry of Health guidelines.

Murine chronic pneumonia model

The experiment was adapted from a model established for *P. aeruginosa* (Bragonzi et al., 2009). 1×10^{10} cfu/ml exponentially grown *S. aureus* were mixed with 9 ml TS Agar (1.5%, 45 °C) and pipetted forcefully into 150 ml heavy mineral oil (45 °C). After immediate stirring for 6 min, the mixture was chilled to 4 °C under continuous stirring (35 min). Agar beads were harvested, washed 6× with PBS and those with 100–200 μm of diameter were selected microscopically. The trachea of anesthetized mice was exposed by ventral midline incision and instilled with a sterile, flexible 22 G needle attached to a 1 ml syringe. Lungs were challenged with agar beads (2×10^6 cfu, 50 μl). After 14 days lungs were excised, homogenized and serial dilutions were plated for cfu counting.

Statistics

Significance was determined by the paired or unpaired two-tailed Student's *t*-test, Mann–Whitney Test, Chi-Square Test, or Spearman's rank correlation using GraphPad Prism Version 6. $P \leq 0.05$ was considered significant.

Details of the further phenotypic assays (hemolysis, susceptibility, LDH-release assay, biofilm formation) are provided in the online supplement.

Results

Long-term persistence of isogenic S. aureus in the airways of CF patients

From two independent CF centers with a total number of 155 patients, we identified 71 patients (46%) with continuous recovery of *S. aureus* from the airways for >5 years. The mean age of the patients at the time of the late isolate was 21.8 years (range 6.3–42.3 years). To assess whether persistence occurred due to isogenic or